Nutritional influences on arsenic toxicity in Bangladeshi men and women: interplay between one-carbon metabolism, arsenic, and epigenetics

Caitlin G. Howe

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ABSTRACT

Nutritional influences on arsenic toxicity in Bangladeshi men and women: interplay between one-carbon metabolism, arsenic, and epigenetics

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Background: In Bangladesh, more than 57 million individuals are exposed to arsenic-contaminated drinking water at concentrations that exceed the World Health Organization guideline for safe drinking water, which is 10 µg/L. Arsenic is a human carcinogen, which has also been associated with numerous non-cancer outcomes, including cardiovascular disease. For many arsenic-related health outcomes, susceptibility differs by sex, with some outcomes preferentially afflicting males and others females. Although reducing exposure to arsenic-contaminated drinking water is the primary strategy for preventing arsenic toxicity, cancer risks remain elevated decades after arsenic exposure has been reduced. Therefore, public health approaches which complement arsenic remediation efforts are needed. One potential set of strategies includes nutritional interventions. Deficiencies in one-carbon metabolism (OCM) nutrients can cause hyperhomocysteinemia (HHcys), which has been associated with adverse health outcomes, including cancers and cardiovascular disease. In Bangladesh, the prevalence of HHcys is quite high and differs by sex (63% among men, 26% among women). Nutrients involved in the OCM pathway may also protect against arsenic toxicity. Two potential mechanisms include: 1) by enhancing arsenic metabolism and 2) by preventing/reversing arsenic-induced epigenetic dysregulation.

Arsenic metabolism facilitates urinary arsenic elimination and depends on two sequential *S*-adenosylmethionine (SAM)-dependent methylation steps, which yield the mono- and dimethyl arsenical species (MMA and DMA, respectively) and *S*-adenosylhomocysteine (SAH), a potent inhibitor of most methyltransferases. SAM is synthesized via OCM, a pathway with many nutritional influences, including folate and cobalamin. There is substantial evidence from experimental studies that the OCM pathway is important for facilitating arsenic metabolism and elimination. However, the relationships between SAM, SAH, and arsenic methylation may be particularly complex in populations exposed continuously to arsenic, because 1) the arsenic metabolites compete for methylation, since each methylation step is catalyzed by the arsenic (+3) methyltransferase and requires a methyl group from SAM, and 2) folate and cobalamin nutritional status may vary between individuals.

Although the mechanisms mediating arsenic toxicity remain largely unclear and are likely multifactorial, there is increasing evidence that arsenic induces epigenetic dysregulation, including alterations in both DNA methylation and posttranslational histone modifications (PTHMs), and these effects may differ by sex. Arsenic has also been shown to alter gene expression in a sex-dependent manner. However, the sex-specific effects of arsenic on PTHMs and gene expression have not been confirmed in a large epidemiological study. Since many of the enzymes involved in epigenetic regulation, including DNA methyltransferases and lysine histone methyltransferases, depend on SAM, epigenetic modifications are also influenced by OCM. Previous studies have demonstrated that nutritional methyl donors involved in the OCM pathway buffer against/modify toxicant-induced alterations in DNA methylation. This may also be true for arsenic-induced alterations in PTHMs. However, the relationships between OCM indices and PTHMs have not been characterized in arsenic-exposed populations.

Objectives: We had five main objectives: 1) to examine the relationships between SAM, SAH, and arsenic methylation capacity, and potential effect modification by folate and cobalamin nutritional status; 2) to characterize a specific cleavage product of histone H3, which we identified in human peripheral blood mononuclear cells (PBMCs) in our early analyses of PTHMs; 3) to evaluate the effects of arsenic exposure and arsenic removal on three candidate PTHMs (di- and tri-methylation at lysine 36 of histone H3 (H3K36me2 and H3K36me3, respectively) and di-methylation at lysine 79 of histone H3 (H3K79me2)), which were selected because they are dysregulated in cancers and are altered by arsenic and/or nutritional methyl donors *in vitro*; 4) to examine associations between arsenic exposure and gene-specific DNA methylation and mRNA expression, particularly for genes involved in pathways implicated in arsenic toxicity; and 5) to characterize the relationships between OCM indices and our three candidate PTHMs, and the effect of folic acid (FA) supplementation on these same PTHMs. For objectives 3-5, we also examined potential differences by sex.

Methods: To address these objectives, we used data from three epidemiological studies of arsenic-exposed Bangladeshi adults: 1) the Folate and Oxidative Stress (FOX) study, a cross-sectional study of healthy individuals; 2) the Folic Acid and Creatine Trial (FACT), a randomized placebo-controlled trial (duration 24 weeks) in which healthy participants received an arsenic-removal water filter at baseline and were also randomized to one of five nutrition intervention arms: placebo, 400 µg FA/day (FA400), 800 µg FA/day (FA800), 3 g creatine/day (Creatine), and Creatine + FA400; and 3) the Bangladesh Vitamin E and Selenium Trial (BEST), a randomized placebo-controlled trial (duration 6 years) in which individuals with arsenicosis were randomized to one of four nutrition intervention arms: placebo, vitamin E (alpha-

tocopheral, 100 mg/day), selenium (*L*-selenomethionine, 200 µg/day), or a combination of vitamin E and selenium.

In Chapter 3, we examined associations between blood SAM and SAH and the proportion (%) of each arsenic metabolite, measured in blood and urine, among FOX participants. We further examined if these associations differed within strata of folate and/or cobalamin nutritional status. In Chapter 4, we characterized a specific cleavage product of histone H3, which we identified in human PBMCs from a subset of FACT participants (n = 32). We also determined the prevalence of H3 cleavage in these samples and the impact of H3 cleavage on the measurement of downstream PTHMs. In Chapter 5, we presented sex-specific associations between pre-intervention measures of blood arsenic and creatinine-adjusted urinary arsenic (uAs_{Cr}) and PTHMs, measured in PBMCs collected from FACT participants (n = 317). We also evaluated whether PTHMs were stable for the 12 week duration after FACT participants received arsenic-removal water filters (n = 60 from placebo group). In Chapter 6, we presented associations between pre-intervention uAs_{Cr} and gene-specific DNA methylation (whole blood, n = 400) and mRNA expression (PBMCs, n = 1799) for 47 candidate genes involved in arsenic metabolism, OCM, epigenetic regulation, DNA repair, or tumor suppression/oncogenesis, using baseline-collected samples from BEST participants. We also evaluated these associations separately by sex. In Chapter 6, we examined sex-specific associations between baseline circulating concentrations of OCM indices, including folate, cobalamin, choline, betaine, and homocysteine, and PTHMs measured in PBMCs collected from FACT participants (n = 324). We also evaluated whether FA400 (n = 106), compared with placebo (n = 60), for a duration of 12 weeks increased global levels of PTHMs.

Results: We observed that folate and cobalamin nutritional status significantly modified associations between SAM and the % arsenic metabolites, as hypothesized (Chapter 3). Among folate and cobalamin deficient individuals, SAM was positively associated with the %MMA, and negatively associated with the %DMA, in blood. In Chapter 4, we determined that H3 cleavage was evident in one third of the FACT PBMC samples examined. We further demonstrated that H3 cleavage impacts the measurement of certain PTHMs. In Chapter 5, we reported that biomarkers of arsenic exposure were associated with H3K36me2 in a sex-dependent manner. In particular, uAs_{Cr} was positively associated with H3K36me2 among men, but not women. Furthermore, the use of arsenic-removal water filters was associated with significant reductions in H3K36me2 over a 12 week period, but this did not differ by sex. We also observed that uAs_{Cr} was associated with the methylation and expression of several genes involved in OCM, epigenetic regulation, DNA repair, and tumor suppression, and many of these associations differed by sex (Chapter 6). The associations between several OCM indices and PTHMs were also sex-dependent (Chapter 7). Specifically, choline was positively associated with H3K36me2 among men only, while cobalamin was positively associated with H3K79me2 among women only. However, FA400 for 12 weeks did not alter global levels of the PTHMs examined.

Conclusions: Given that cancer risks remain elevated decades after arsenic exposure has ceased, public health interventions which complement arsenic remediation efforts are needed. Nutritional interventions may be one promising approach. Previous studies have observed that a higher %MMA, and a lower %DMA, in urine is associated with an increased risk of developing adverse health outcomes. Our finding that SAM was positively associated with %MMA, and negatively associated with %DMA, among individuals deficient for folate and cobalamin contributes

additional evidence that nutritional status may explain some of the inter-individual differences in arsenic methylation capacity and, consequently, in susceptibility to arsenic toxicity.

Our observation that arsenic exposure was positively associated with global levels of H3K36me2 among men, but not women, and that arsenic was associated with gene-specific DNA methylation and mRNA expression in a sex-dependent manner, adds to a growing literature that arsenic induces epigenetic dysregulation differentially by sex. Furthermore, these findings suggest that this may have functional consequences, such as alterations in mRNA expression, including for genes involved in pathways implicated in arsenic toxicity. While it is tempting to speculate that this may explain some of the sex differences in susceptibility to arsenic toxicity, the clinical implications of our findings will require additional study.

We also provided the first evidence from an arsenic-exposed population that choline and cobalamin are associated with PTHMs (H3K36me2 and H3K79me2, respectively) in a sex-dependent manner, and that 12 weeks' supplementation with FA, at a dose based on the recommended dietary allowance for folate, does not significantly alter global levels of H3K36me2, H3K36me3, or H3K79me2 in human PBMCs. Previous studies have shown that nutrients in the OCM pathway protect against toxicant-induced alterations in DNA methylation. Our findings suggest that some OCM nutrients, particularly choline and cobalamin, may also influence PTHMs in human PBMCs. These findings lay the groundwork for future studies which further examine whether these nutrients can protect against or modify arsenic-induced alterations in PTHMs.

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LIST OF ABBREVIATIONS

400FA	400 µg folic acid per day
5,10-mTHF	5,10-methylene-tetrahydrofolate
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
5-mTHF	5-methyl-tetrahydrofolate
5'-UTR	5' untranslated region
800FA	800 µg folic acid per day
ac	Acetylated
AHCY	*S*-adenosylhomocysteine hydrolase
AI	Adequate intake
APL	Acute promyelocytic leukemia
Arg.	Argentina
As	Arsenic
As_2O_3	Arsenic trioxide
As^{III}	Arsenite
As^{V}	Arsenate
AS3MT	Arsenic (+3 oxidation state) methytransferase
bAs	Blood arsenic
bDMA	Blood dimethyl arsenical species
BEST	Bangladesh Vitamin E and Selenium Trial
BHMT	Betaine homocysteine methyltransferase
bInAs	Blood inorganic arsenical species
bMMA	Blood monomethyl arsenical species
bSe	Blood selenium
C_2H_7AsI	Dimethylarsine iodide
CAE	Cumulative arsenic exposure
CBS	Cystathionine-β-synthase
CGI	Cytosine-guanine dinucleotide island
CH_3AsI_2	Diiodomethylarsine
CHDH	Choline dehydrogenase
CIN	Cervical intraepithelial neoplasia
CoA	Coenzyme A
CpG	Cytosine-guanine dinucleotide
CTD	Comparative Toxicogenomics Database
CVD	Cardiovascular disease
d	Days
Develop. Delay	Developmental delay

DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DMA	Dimethyl arsenical species
DMA^{III}	Trivalent dimethyl arsenic
DMA^{V}	Pentavalent dimethyl arsenic
DMA:MMA	Ratio of dimethyl to monomethyl arsenical species
%DMA	Proportion of dimethyl arsenic species
DMG	Dimethyl glycine
DNMT	DNA methyltransferase
E2	Estradiol
ESC	Embryonic stem cell
F	Female
FA	Folic acid
FACT	Folic Acid and Creatine Trial
FOX	Folate and Oxidative Stress Study
GAA	Guanidinoacetate
GAMT	Guanidinoacetate *N*-methyltransferase
GNMT	Glycine *N*-methyltransferase
GSH	Glutathione
GSS	Glutathione synthetase
GSSG	Glutathione disulfide
H3K4	Histone H3 lysine 4
H3K9me2	Histone H3 lysine 9 di-methylation
H3K36	Histone H3 lysine 36
H3K36me1	Histone H3 lysine 36 mono-methylation
H3K36me2	Histone H3 lysine 36 di-methylation
H3K36me3	Histone H3 lysine 36 tri-methylation
H3K79me1	Histone H3 lysine 79 mono-methylation
H3K79me2	Histone H3 lysine 79 di-methylation
Hcys	Homocysteine
HEALS	Health Effects of Arsenic Longitudinal Study
HHcys	Hyperhomocysteinemia
HR	Hazards ratio
InAs	Inorganic arsenical species
%InAs	Proportion of inorganic arsenical species
IF	Intrinsic factor
IQR	Inter-quartile range
KDM	Lysine histone demethylase
KHMT	Lysine histone methyltransferase
Log	Natural log transformation

M	Male
MAT1A	Methionine adenosyltransferase 1 (hepatic)
MAT2A	Methionine adenosyltransferase 2 subunit alpha (non-hepatic)
MAT2B	Methionine adenosyltransferase 2 subunit beta (non-hepatic)
MDD	Methyl deficient diet
MMA	Monomethyl arsenical species
MMA^{III}	Trivalent monomethyl arsenic
MMA^{V}	Pentavalent monomethyl arsenic
MMA:InAs	Ratio of monomethyl to inorganic arsenical species
%MMA	Proportion of monomethyl arsenical species
me1	Mono-methylated
me2	Di-methylated
me3	Tri-methylated
Met	Methionine
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
MTRR	Methionine synthase reductase
N_Shore	North shore
$NaAsO_2$	Sodium arsenite
Na_3AsO_4	Sodium arsenate
NHEJ	Non-homologous end joining
NTD	Neural tube defect
OCM	One-carbon metabolism
OR	Odds ratio
PABA	4-aminobenzoic acid
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cell
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine *N*-methyltransferase
ph	Phosphate
PTHM	Posttranslational histone modification
PVD	Peripheral vascular disease
RBC	Red blood cell
RDA	Recommended dietary allowance
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SAM:SAH	Ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine
SNP	Single nucleotide polymorphism
TET	Ten-eleven translocation methylcytosine dioxygenase

THF	Tetrahydrofolate
TRX	Thioredoxin
TSS200	Within 200 basepairs of a transcription start site
uAs	Urinary arsenic
uAs_{Cr}	Urinary arsenic adjusted for urinary creatinine
uCr	Urinary creatinine
uDMA	Urinary dimethyl arsenical species
uInAs	Urinary inorganic arsenical species
uMMA	Urinary monomethyl arsenical species
UL	Upper limit
U.S.	United States
w	Weeks
wAs	Water arsenic
WBC	White blood cell
y	Years

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DEDICATION

For my family

CHAPTER ONE

Statement of hypotheses

In Bangladesh, approximately 57 million individuals are exposed to arsenic at concentrations that exceed the World Health Organization guideline for safe drinking water, which is 10 μg/L [1]. This is a critical public health issue, because exposure to arsenic has been associated with numerous diseases, including both cancer and non-cancer outcomes, with susceptibility often differing by sex (reviewed in [2]). Although eliminating exposure to arsenic-contaminated drinking water is the primary strategy for reducing arsenic toxicity, cancer risks remain elevated decades after arsenic exposure has ceased [3]. Therefore, complementary approaches will be needed to reduce disease burden in populations that have already been exposed to arsenic-contaminated drinking water.

Nutritional interventions may offer one set of complementary approaches. One-carbon metabolism (OCM), a biochemical pathway with many nutritional influences, including folate, cobalamin, choline, and betaine, may affect arsenic toxicity through multiple mechanisms. Two potential mechanisms include: 1) by enhancing arsenic metabolism and 2) by preventing or reversing arsenic-induced epigenetic dysregulation, including alterations in posttranslational histone modifications (PTHMs) (**Figure 1**). Since arsenic metabolism facilitates urinary arsenic excretion [4], and since epigenetic dysregulation is one proposed mechanism by which arsenic causes cancer [5], a better understanding of these mechanisms may inform the development of future interventions which reduce arsenic toxicity. This dissertation is an epidemiological investigation of the interplay between OCM, arsenic, and epigenetics.

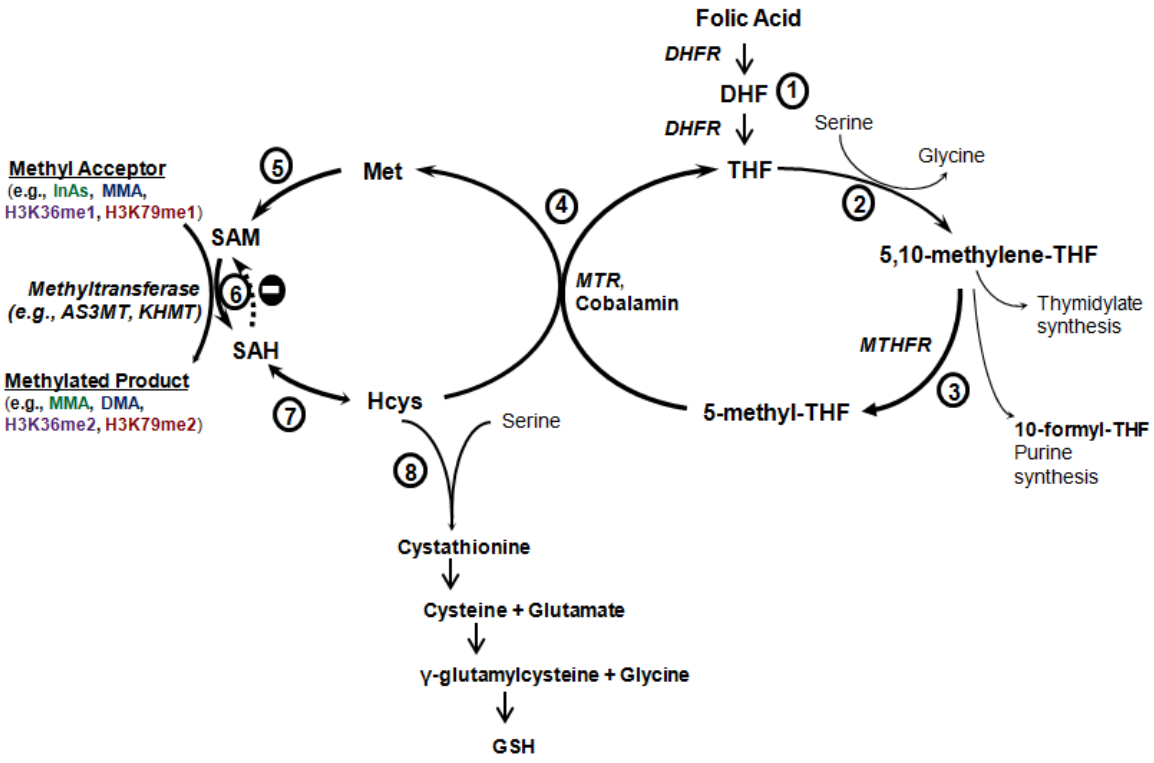

Figure 1. One-carbon metabolism and the transsulfuration pathway. (1) Dietary folic acid is reduced to dihydrofolate (DHF) and tetrahydrofolate (THF) by dihydrofolate reductase (DHFR). (2) The β-carbon of serine is transferred to THF by serine hydroxymethyltransferase, forming 5,10-methenyl-THF and glycine. (3) At a major branch point between transmethylation reactions and nucleotide biosynthesis, 5,10-methenyl-THF can be reduced to 5,10-methylene-THF, which can be further reduced to 5-methyl-THF by methylenetetrahydrofolate reductase (MTHFR). (4) In a reaction catalyzed by cobalamin-dependent methionine synthase (MTR), the methyl group of 5-methyl-THF is transferred to homocysteine (Hcys), generating methionine (Met) and regenerating THF. Alternatively, betaine Hcys methyltransferase (not shown) can transfer a methyl group from betaine for the remethylation of Hcys; betaine can be obtained from the diet or derived from choline. (5) Methionine adenosyltransferase activates methionine to form *S*-adenosylmethionine (SAM). (6) SAM serves as a universal methyl donor for numerous acceptors, including inorganic arsenic (InAs) and monomethylarsonic acid (MMA) as well as lysine residues of histone proteins, such as lysines 36 and 79 of histone H3 (H3K36 and H3K79, respectively), which are catalyzed by various SAM-dependent lysine histone methyltransferases (KHMTs). Examples of methyl acceptors and their respective methylated products are shown in matching colors. (7) Upon donating a methyl group, SAM is converted to *S*-adenosylhomocysteine (SAH), a potent inhibitor of most SAM-dependent methyltransferases. SAH is hydrolyzed to generate Hcys, which is then either used to regenerate Met or is directed to (8) the transsulfuration pathway through which it is ultimately catabolized to cystathionine for the synthesis of glutathione (GSH). Other abbreviations used: AS3MT, arsenic (+3 oxidation state) methyltransferase; DMA, dimethylarsinic acid; H3K36me1, mono-methylation at lysine 36 of histone H3; H3K36me2, di-methylation at lysine 36 of histone H3; H3K79me1, mono-methylation at lysine 79 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3

Hypothesis 1

We hypothesize that blood *S*-adenosylmethionine (SAM) will be negatively associated with the proportion (%) of inorganic arsenical species (InAs) in blood and urine, while blood *S*-adenosylhomocysteine (SAH) will be associated with an arsenic metabolite profile indicative of reduced arsenic methylation capacity (i.e., ↑%InAs, ↑% monomethyl arsenical species (MMA), and ↓% dimethyl arsenical species (DMA) in blood and urine). We further hypothesize that folate and cobalamin nutritional status will modify the associations between SAM and the methylated arsenic metabolites (%MMA and %DMA).

Specific Aim 1a. SAM, SAH, and arsenic methylation

We will examine associations between whole blood SAM and SAH concentrations and arsenic metabolites, measured as the % of each metabolite (InAs, MMA, and DMA) in total blood or urine arsenic (bAs and uAs, respectively), using samples from the Folate and Oxidative Stress (FOX) Study, a cross-sectional study of Bangladeshi adults (n = 378) exposed to a wide range of water arsenic concentrations (0 to 700 µg/L).

Specific Aim 1b. Folate and cobalamin nutritional status, SAM, and arsenic methylation

Using samples from the FOX study, we will evaluate whether folate and/or cobalamin nutritional status modify associations between SAM and the methylated arsenic metabolites (%MMA and %DMA), measured in blood and urine. Folate and cobalamin nutritional status will be determined using plasma folate and cobalamin cutoffs (9 nmol/L and 151 pmol/L, respectively [6]).

The findings of Aims 1a and 1b are reported in Chapter 3.

Characterizing enzymatic cleavage of histone H3

In our early analyses of PTHMs, we identified a specific cleavage product of histone H3 in human peripheral blood mononuclear cells (PBMCs), which were collected from participants enrolled in the Folic Acid and Creatine Trial (FACT). We describe this cleavage product, the prevalence of H3 cleavage in human PBMC samples, and the impact of H3 cleavage on the measurement of downstream PTHMs.

These findings are reported in Chapter 4.

Hypothesis 2

We hypothesize that arsenic induces sex-specific alterations in global levels of three candidate PTHMs: histone H3 lysine 36 di- and tri-methylation (H3K36me2 and H3K36me3, respectively) and histone H3 lysine 79 di-methylation (H3K79me2), which were selected because they are influenced by arsenic and/or nutritional methyl donors in experimental models [7-10] and are dysregulated in cancers [11-16]. H3K36me2 was also selected based on a previous finding from our group that among men only, arsenic is positively associated with DNA methylation in the promoter region of lysine demethylase 2B, a histone demethylase that specifically targets H3K36me2. Furthermore, H3K79me2 was also selected, because it regulates the expression of Tet1, which converts 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) [17, 18]. We have previously observed that both 5-mC and 5-hmC are altered by arsenic in a sex-dependent manner [19]. We further hypothesize that arsenic exposure is associated with gene-specific DNA methylation and mRNA expression in a sex-dependent manner.

Specific Aim 2a. Sex-specific effects of arsenic on PTHMs

We will examine associations between pre-intervention measures of bAs and creatinine-adjusted uAs (uAs_{Cr}) and H3K36me2, H3K36me3, and H3K79me2, measured in histones isolated from PBMCs collected from a subset of FACT participants at baseline (*n* = 317). FACT is a randomized placebo-controlled trial of folic acid (FA) and/or creatine supplementation in arsenic-exposed Bangladeshi adults. We will further determine if the associations between arsenic exposure and PTHMs differ by sex.

Specific Aim 2b. Stability of PTHMs after reductions in arsenic exposure

We will evaluate whether H3K36me2, H3K36me3, and H3K79me2 are altered after the provision of arsenic-removal water filters. This will be examined using PBMCs collected at baseline and week 12 from a subset of FACT participants randomized to the placebo group (*n* = 60), who were provided with arsenic-removal water filters but did not receive a nutritional intervention. We will further examine whether alterations in PTHMs due to arsenic-removal water filter use differ by sex.

The findings of Aims 2a and 2b are reported in Chapter 5.

Specific Aim 2c. Sex-specific associations between arsenic exposure and gene-specific DNA methylation and mRNA expression levels

Using pre-intervention samples from the Bangladesh Vitamin E and Selenium Trial (BEST), a randomized trial of vitamin E and/or selenium supplementation in Bangladeshi adults with arsenicosis, we will examine whether uAs_{Cr} is associated with gene-specific DNA methylation (whole blood, *n* = 400) and mRNA expression (PBMCs, *n* = 1799). We will use a

candidate gene approach, selecting genes involved in OCM, arsenic metabolism, epigenetic regulation, DNA repair, and tumor suppression/oncogenesis, as these pathways are involved in arsenic metabolism or have been implicated in arsenic toxicity. The Comparative Toxicogenomics Database will be used to identify potential targets of arsenic. We will also select a subset of genes involved in these pathways which have not previously been examined in relation to arsenic exposure. We will further examine potential differences by sex.

The findings of Aim 2c are reported in Chapter 6.

Hypothesis 3

We hypothesize that nutritional methyl donors and related cofactors involved in the OCM pathway, including folate, cobalamin, choline, and betaine, will be positively associated with PTHMs (H3K36me2, H3K36me3, and H3K79me2), and that homocysteine, an indicator of reduced methylation capacity, will be inversely associated with these PTHMs. Furthermore, we hypothesize that FA supplementation will increase global levels of these PTHMs. Additionally, we hypothesize that these relationships may differ by sex.

Specific Aim 3a. Associations between OCM indices and PTHMs

Using pre-intervention samples from the FACT study (n = 324), we will examine associations between circulating concentrations of OCM indices and global levels of H3K36me2, H3K36me3, and H3K79me2, measured in PBMCs. Furthermore, we will assess whether any of these associations differ by sex.

Specific Aim 3b. Influence of FA supplementation on PTHMs

Using samples from the FACT study, we will investigate whether FA supplementation (400 μg/day for 12 weeks) (n = 107), compared with placebo (n = 60), increases global levels of H3K36me2, H3K36me3, and H3K79me2, measured in PBMCs. We will also examine potential differences by sex.

The findings of Aims 3a and 3b are reported in Chapter 7.

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CHAPTER TWO

Background

A. Overview of one-carbon metabolism and the transsulfuration pathway

1. One-carbon metabolism

One-carbon metabolism (OCM) consists of a series of oxidation and reduction reactions that involve the transfer of one-carbon units via folate [1]. These one-carbon units are primarily derived from formate and the hydroxymethyl group of serine [2]. The reactions of OCM are highly compartmentalized between the cytoplasm, mitochondria, and nucleus and are involved in many essential reactions, including the biosynthesis of thymidylate and purines and numerous transmethylation reactions [1, 3].

Nucleic acid synthesis

The *de novo* synthesis of deoxythymidine monophosphate requires the transfer of a one-carbon group from folate, in the form of 5,10-methylene-tetrahydrofolate (5,10-mTHF), to the 5' position of deoxyuridine monophosphate [1, 3]. This primarily occurs in the cytoplasm and is catalyzed by thymidylate synthase [1, 3]. In folate limiting conditions, thymidylate synthesis is impaired [4]. As a result, uracil misincorporation into DNA occurs, which can lead to chromosomal breaks and genomic instability [4].

Purine ring synthesis is largely dependent on purine recycling via the purine nucleotide salvage pathway [5]. However, *de novo* purine synthesis is critical during periods of rapid cell division, such as embryogenesis [6], and when purine nucleotides are limiting [5, 7]. *De novo* purine synthesis depends on the donation of two formate groups, provided by two molecules of

folate, in the form of 10-formyl-tetrahydrofolate, which become carbons 2 and 8 of the newly synthesized purine ring [3, 5].

Methylation reactions

S-adenosymethionine (SAM) is involved in more than 100 different methylation reactions [8], which are important for diverse biological processes, including stabilization of DNA, RNA, and proteins; small molecule biosynthesis; cell signaling; and inactivation or elimination of small molecules, including xenobiotics [9]. SAM is therefore considered the universal methyl donor [10]. Each SAM-dependent methyltransferase transfers a methyl group from SAM to its respective substrate, which results in the methylated product and *S*-adenosyhomocysteine (SAH) (**Figure 1**). SAH is a potent inhibitor of most SAM-dependent methyltransferases [9]. Thus, SAM and SAH are considered indices of methylation capacity [11-14].

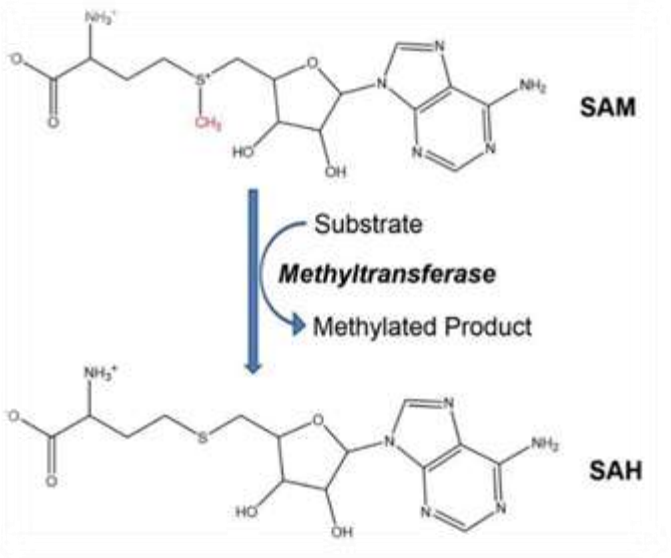

Figure 1. *S*-adenosylmethionine and *S*-adenosylhomocysteine. *S*-adenosylmethionine (SAM) can donate a methyl group (shown in red) to a large number of different substrates. Upon donating this methyl group, SAM is converted to *S*-adenosylhomocysteine (SAH), a potent inhibitor of most SAM-dependent methyltransferases.

SAM synthesis is catalyzed by methionine adenosyltransferase and depends on the activation of methionine (Met) by ATP [15, 16]. Met can be obtained from the diet or synthesized via the methylation of homocysteine (Hcys) in a reaction catalyzed by one of two enzymes: 1) Met synthase (MTR), which utilizes cobalamin as a cofactor and requires a methyl donation from folate in the form of 5-methyl-tetrahydrofolate (5-mTHF) or 2) betaine Hcys methyltransferase (BHMT), which requires a methyl donation from betaine (**Figure 2**) [17]. Although both pathways contribute to Met synthesis, *MTR* is expressed ubiquitously [18], while *BHMT* expression is mainly confined to the kidney and liver [19]. Given their importance in numerous methylation reactions, intracellular SAM and SAH concentrations are very tightly controlled [20]. In part, this occurs through negative feedback via long-range allosteric regulation. For example, SAM inhibits methylenetetrahydrofolate reductase (MTHFR), which irreversibly converts 5,10-mTHF to 5-mTHF [21]. SAM also inhibits BHMT [22]. Thus, SAM downregulates its own synthesis by simultaneously reducing the production of 5-mTHF and the amount of betaine utilized for the remethylation of Hcys (Figure 2).

Since SAH is a potent inhibitor of most SAM-dependent methyltransferases [9], the elimination of SAH is also important for maintaining efficient methylation capacity. SAH can be hydrolyzed to Hcys by SAH hydrolase [23]. However, this reaction is reversible and strongly favors SAH synthesis [23]. Therefore, SAH will readily accumulate, leading to the inhibition of multiple methylation reactions, unless Hcys is rapidly eliminated by 1) remethylation to Met by either the folate- and cobalamin-dependent or the betaine-dependent pathway or 2) catabolism via the transsulfuration pathway [23].

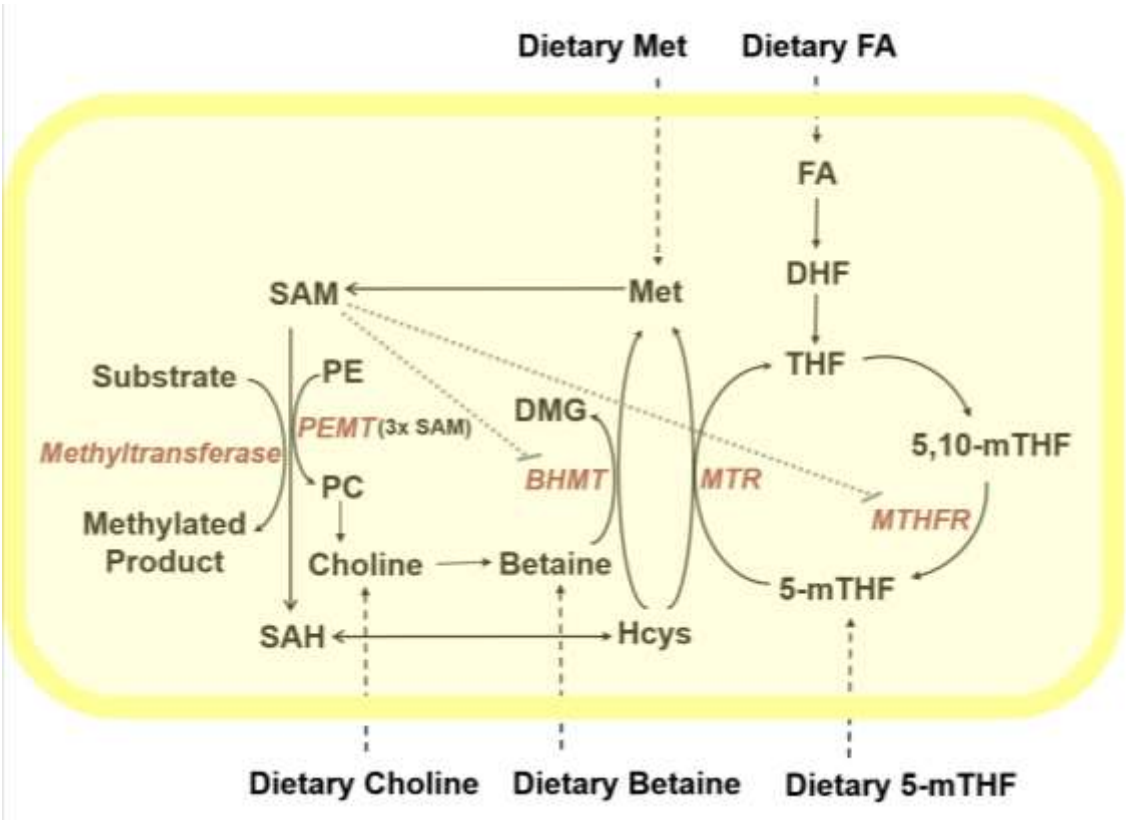

Figure 2. Simplified overview of one-carbon metabolism. *S*-adenosylmethionine (SAM) donates a methyl group to one of its numerous substrates to form the methylated product and *S*-adenosylhomocysteine (SAH), which can be hydrolyzed to homocysteine (Hcys). However, this reaction is reversible and favors SAH synthesis. The regeneration of SAM depends on methionine (Met), which can be obtained from the diet or synthesized endogenously via the remethylation of Hcys. This is either catalyzed by 1) methionine synthase (MTR), a cobalamin-dependent enzyme that requires a methyl donation from 5-methyl-tetrahydrofolate (5-mTHF), or 2) betaine homocysteine methyltransferase (BHMT), which requires a methyl donation from betaine. Once betaine donates a methyl group, it forms dimethylglycine (DMG). 5-mTHF can be obtained directly from the diet, or it can be derived from folic acid (FA). FA must be reduced to dihydrofolate (DHF), then tetrahydrofolate (THF), before receiving a methyl group from serine to form 5,10-methylene-tetrahydrofolate (5,10-mTHF). 5,10-mTHF is then converted to 5-mTHF by methylene tetrahydrofolate reductase (MTHFR). Betaine can either be obtained from the diet or derived from the irreversible oxidation of choline, which can also be acquired from the diet or synthesized endogenously. *De novo* choline synthesis involves three sequential methylation reactions, catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT), which converts phosphatidylethanolamine (PE) to phosphatidylcholine (PC). The synthesis of one molecule of PC requires three molecules of SAM and generates three molecules of SAH. To ensure tight control of intracellular SAM and SAH concentrations, many enzymes involved in one-carbon metabolism are regulated through long-range allosteric interactions. For example, SAM inhibits both BHMT and MTHFR and thereby downregulates its own synthesis.

Folate

Folate is a general term used to describe a family of many different folate metabolites, which can either directly donate one-carbon groups or serve as intermediates by transferring one-carbon groups to other folate metabolites. Folic acid (FA), the synthetic form of folate, is the reference compound. FA is composed of a pteridine (2-amino-4-hydroxypteridine) ring, connected by a methylene bridge to *p*-aminobenzoic acid (PABA), which in turn is joined by a peptide linkage to a glutamate residue [21] (**Figure 3**). Naturally occurring folates follow the same structure, but with three major differences: 1) the pteridine ring is reduced, 2) one-carbon substitutions are present at the N5 and/or N10 position, and 3) PABA is connected to a polyglutamyl tail, rather than to a single glutamate residue [21].

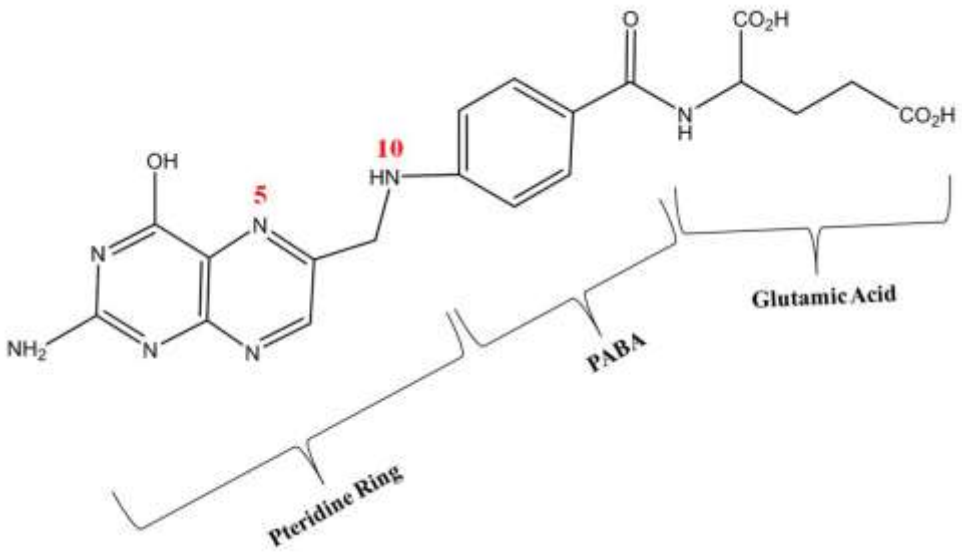

Figure 3. Structure of folic acid. Folic acid consists of three major moieties: a pteridine ring, *p*-aminobenzoic acid (PABA), and a glutamic acid residue. Naturally occurring folates differ in that the pteridine ring is reduced, one-carbon substitutions can occur at the N5 and/or N10 positions (indicated in red), and they have polyglutamyl tails, which are typically 4 to 10 glutamate residues in length.

Folate is mainly absorbed in the proximal jejunum, where naturally occurring folates must be cleaved to monoglutamate species prior to being transported into the enterocyte, primarily by the reduced folate carrier or the proton-coupled folate transporter [24]. FA is more readily absorbed than naturally occurring folates, in part because FA does not need to be cleaved and thus can be directly transported into the enterocyte [21]. After being reduced in the enterocyte, FA is converted with naturally occurring folates to 5-mTHF [21], the predominant form of circulating folate [21].

In the United States, the recommended dietary allowance (RDA) for folate is 400 µg dietary folate equivalents/day for adults [25]. This is comparable to 400 µg natural folate from food sources, 240 µg FA taken with food, or 200 µg FA taken on an empty stomach [25]. Food sources particularly rich in natural folates include leafy greens and other vegetables, such as Brussels sprouts and asparagus, as well as beef liver, beans, and nuts [25]. In countries with mandatory FA fortification programs, fortified staple foods are rich sources of FA. In 1998, the United States mandated fortification of cereals, breads, pastas, and other grain products with FA [25]. Thus, with the exception of certain subgroups, the prevalence of folate deficiency in the United States has remained very low (<5%) since 1998 [26]. However, many countries do not have mandatory FA fortification, and have a high prevalence of folate deficiency. For example, in Bangladesh the prevalence of folate deficiency (plasma folate <9 nmol/L [27]) is estimated to be as high as 57% for men and 39% for women [28].

The upper tolerable limit (UL) for FA is 1 mg/day. This was originally established based on concerns that FA could mask cobalamin deficiency, since FA supplementation can correct megaloblastic anemia, the main clinical symptom of cobalamin deficiency, without correcting other potential consequences, such as neurological damage [25]. These concerns largely arose in

response to early case studies which reported neurological symptoms in cobalamin deficient individuals treated with FA [29].

Cobalamin

In mammals, cobalamin, also known as vitamin B12, exists in two main active forms: methylcobalamin and 5'-deoxyadenosylcobalamin [21]. These are the predominant forms of cobalamin in the serum and cytosol, respectively [30]. Methylcobalamin is a cofactor for MTR, while 5'-deoxyadenosylcobalamin is a cofactor for methylmalonyl coenzyme A (CoA) mutase, which converts methylmalonyl CoA to succinyl CoA, an intermediate in the citric acid cycle [21]. In addition to its role in SAM synthesis, MTR is critical for regenerating tetrahydrofolate (THF) from 5-mTHF [21]. Therefore, cobalamin deficiency can lead to a functional folate deficiency due to a phenomenon called the "methyl trap", where in the absence of cobalamin, folate becomes trapped as 5-mTHF, since MTR is thus unable to convert 5-mTHF to THF (Figure 2). Consequently, an intracellular deficiency in THF develops, leading to the impairment of downstream processes, such as purine and thymidylate synthesis [21].

In the United States, the RDA for cobalamin is 2.4 µg/day for adults [31]. Major dietary sources include meat, eggs, and dairy [31]. In high-income countries, many cereals also contain cobalamin as a result of voluntary fortification [32, 33]. The main type of cobalamin used in fortified foods and supplements is cyanocobalamin, which is easily absorbed and can be converted endogenously to the biologically active forms methylcobalamin and 5'deoxyadenosylcobalamin [31]. There is currently no UL for cobalamin [31], since randomized trials that administered doses as high as 0.4 and 1 mg/day did not observe toxic side effects [34, 35].

Dietary cobalamin is tightly bound to proteins and must therefore be released by hydrochloric acid in the stomach to be effectively absorbed [36]. However, free cobalamin is very susceptible to denaturation and must bind instantly to haptocorrin, a glycoprotein which protects it until it reaches the duodenum [37]. Once in the duodenum, cobalamin is released and bound by intrinsic factor (IF), [36, 38] another glycoprotein, which is essential for cobalamin's subsequent absorption in the ileum [39]. Upon absorption, cobalamin is released from IF and is thus able to form a complex with transcobalamin II [40], which circulates in the blood and is transported into cells by transcobalamin receptors, which are expressed ubiquitously [41, 42].

Gastric atrophy occurs with aging, leading to decreased production of hydrochloric acid and IF. As a result, the elderly are particularly susceptible to developing cobalamin deficiency [33]. In the United States, the prevalence of cobalamin deficiency is highest among those greater than 60 years old, with 6% classified as deficient (plasma cobalamin <148 pmol/L) and more than 20% classified as marginally depleted (148 pmol/L< plasma cobalamin <221 pmol/L) [33]. Since cobalamin is only found naturally in animal products, vegans and vegetarians are also susceptible to developing cobalamin deficiency [33]. Due to expense, meat is not a major component of the diet in Bangladesh. Consequently, there is a high prevalence of cobalamin deficiency in Bangladesh, even among individuals less than 60 years old, with 8% of men and 13% of women between the ages of 28 and 49 classified as deficient (plasma cobalamin <151 pmol/L [27]) [28].

Betaine

Betaine, also known as trimethylglycine, has two major physiological functions: 1) to act as an osmolyte and 2) to serve as a methyl donor for the regeneration of SAM [43]. Due to its

role as a methyl donor, betaine supplementation reduces Hcys concentrations [44-47]. Although the Hcys-reducing effects of betaine are smaller than those observed for FA [48], they are more pronounced in individuals with low plasma folate [49]. Food sources rich in betaine include wheat, shellfish, spinach, and sugar beets [43]. There is currently no RDA or adequate intake (AI) level for betaine.

Studies in animal models [43, 50, 51] and human participants [52, 53] have shown that dietary betaine is rapidly absorbed, then transported into tissues by amino acid transport systems [43], such as amino acid transport system A [54] and the betaine/γ–aminobutyric acid transporter [55, 56]. However, the majority of betaine is synthesized endogenously [43] in a two-step process that occurs in the liver and kidney [57-59], whereby choline is first irreversibly oxidized by choline dehydrogenase to betaine aldehyde, which is subsequently oxidized to betaine in a reaction catalyzed by betaine aldehyde dehydrogenase [60].

Choline

Choline is an essential component of membrane phospholipids and is also required for lipid transport and acetylcholine synthesis [61]. Since choline is a precursor to betaine, it also serves as an important methyl donor, particularly when Met and folate are limiting [61]. Choline can be obtained from the diet or synthesized endogenously. Approximately 70% of endogenous choline is provided by the Kennedy Pathway [62, 63]. The remaining 30% is synthesized *de novo* through a pathway which converts phosphatidylethanolamine (PE) to phosphatidylcholine (PC) through three methylation reactions, which are catalyzed by the SAM-dependent phosphatidylethanolamine *N*-methyltransferase (PEMT) [60] (Figure 2). Consequently, PC

synthesis is one of the largest consumers of SAM [60]. However, choline also contributes to SAM synthesis, since the majority of choline is irreversibly oxidized to betaine in the liver [60].

Although choline is synthesized endogenously in the liver [60, 64] and, to a lesser extent, in the brain [60, 65, 66], it must also be obtained from the diet [60]. Major dietary sources include liver, egg yolks, fish, and dairy products [60, 61]. The main forms of choline obtained through these food sources include free choline and choline esters, such as phosphocholine, glycerophosphocholine, PC, and sphingomyelin [67]. Although there is currently insufficient information to set an RDA for choline [61], the recommended AI is 550 mg/day for adult men and 425 mg/day for adult women who are not pregnant or lactating [61]. These AIs were largely determined based on the amount of choline required to prevent liver damage [60]. Given the hypotensive properties of choline, proposed ULs have also been set for certain subgroups, including an UL of 3.5 g choline/day for women who are pregnant or lactating [60].

Homocysteine and hyperhomocysteinemia

Hcys is a thiol-containing amino acid which is involved in both the transmethylation and the transsulfuration pathways [17]. Plasma Hcys concentrations rise when Hcys accumulates intracellularly [17]. Individuals with plasma Hcys concentrations exceeding 13 μmol/L are classified as having hyperhomocysteinemia (HHcys) [68], which is a risk factor for several adverse health outcomes, including cardiovascular disease [69] and neurological disorders [70]. In human populations, several factors have been associated with elevated plasma Hcys concentrations, including increased age, decreased renal function, and certain genetic variants (reviewed in [17]). Plasma Hcys concentrations are also consistently higher among men

compared with women [17]. Additionally, deficiencies in B vitamins, particularly folate, are important determinants of elevated plasma Hcys concentrations [17].

2. Transsulfuration pathway

The transsulfuration pathway involves the unidirectional catabolism of Hcys for the synthesis of glutathione (GSH) [71, 72], the primary intracellular antioxidant in mammals [71, 72]. This occurs in two major steps: 1) Hcys and serine condense to form cystathionine in a reaction that is catalyzed by cystathionine-β-synthase (CBS) and 2) cystathionine is cleaved by γ-cystathionase to liberate cysteine for GSH synthesis [73]. Vitamin B6, in the form of pyridoxal phosphate, is a required cofactor for each step [73].

B. Overview of arsenic metabolism and toxicity

1. Arsenic and associated health outcomes

Exposure to arsenic-contaminated drinking water is a global problem. Worldwide, it has been estimated that 140 million individuals are exposed to inorganic arsenic (InAs) at concentrations exceeding the World Health Organization guideline for safe drinking water [74], which is 10 μg/L [75]. More than 57 million of these individuals reside in Bangladesh [76]. This is a critical public health issue, because there is substantial evidence that arsenic causes skin, lung, and bladder cancers, ischemic heart disease, and skin lesions (reviewed in [77]). Arsenic has also been associated with kidney, prostate, liver, and pancreatic cancers (reviewed in [77]), peripheral neuropathy [78], decreased intellectual function in children [79], nonmalignant lung disease (reviewed in [80]), diabetes [81-83], and hypertension (reviewed in [84]). In both animal

and human studies, susceptibility to arsenic toxicity has been shown to differ by sex[1], with some outcomes preferentially afflicting males and others females (reviewed in [77]).

Although InAs can exist in four oxidation states (-III, 0, III, and V) [85], in well water it predominately exists in the trivalent (III) and pentavalent (V) states (arsenite and arsenate, respectively) [77].

2. Uptake and metabolism of inorganic arsenic

Almost all ingested InAs is absorbed by the gastrointestinal tract [77, 86]. Although the mechanisms of absorption are still being elucidated, arsenate (As^{V}) competes for uptake by phosphate transporters [87], while arsenite (As^{III}) is likely taken up by several different transporters, including organic anion transporting polypeptides, glucose transporters, and aquaporins 7 and 9 [87]. Once absorbed, InAs is metabolized through a two-step methylation process, which is catalyzed by the SAM-dependent, As^{III} methyltransferase (AS3MT) [88, 89].

Three detailed mechanisms have been proposed for the sequential methylation of InAs to mono- and dimethyl arsenical species (MMA and DMA, respectively). The most commonly accepted pathway is that proposed by Challenger et al., whereby InAs is methylated via an oxidative methylation pathway, in which the valence state of As changes from III to V each time a methyl group is transferred from SAM [90, 91] (**Figure 4**). Two alternative pathways have also been hypothesized. In 2005, Hayakawa et al. described a mechanism in which arsenic is successively methylated, but the valence state does not change with the transfer of a methyl group from SAM [92]; instead, arsenic-GSH complexes form prior to each methylation step [92].

[1]For simplicity, the word "sex" is used. However, in epidemiological studies, observed differences between males and females may be driven by both biological sex, which is determined by sex chromosomes and gonads, and socially-determined factors (i.e., gender-related factors). Therefore, with respect to human populations, "sex" refers to both sex and gender unless otherwise specified.

However, in 2012, Wang et al. provided evidence that arsenic-thiol, not necessarily arsenic-GSH, complexes were the required substrates for arsenic methylation [93]. Despite their differences, the three proposed mechanisms are unified in that AS3MT catalyzes both methylation steps, and each methylation step requires a methyl donation from SAM and is inhibited by SAH. Although the methylation of InAs is thought to primarily occur in the liver, studies in rats have shown that *As3mt* is also expressed in several other tissues, including the lung, bladder, heart, brain, kidney, and adrenal gland [88].

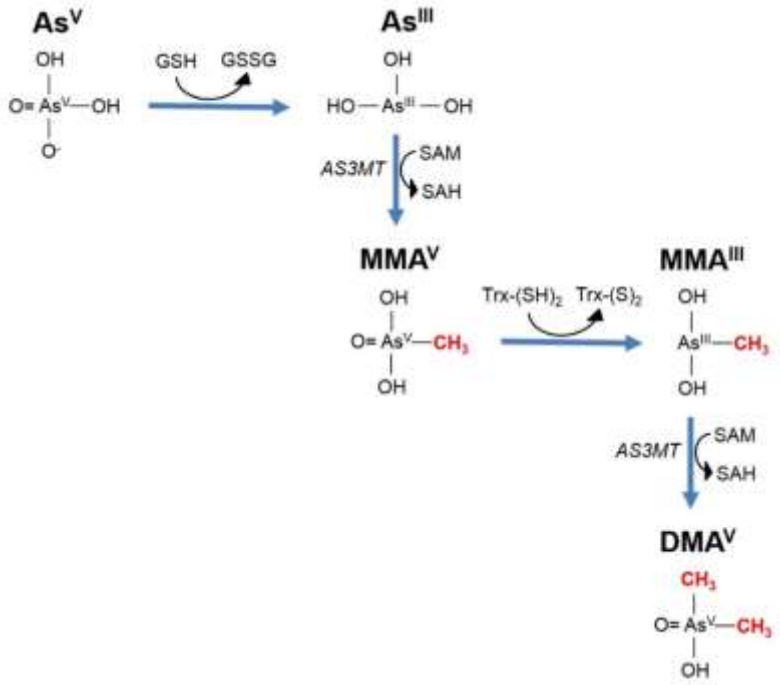

Figure 4. Arsenic metabolism (Challenger pathway). Arsenate (As^{V}) is reduced by glutathione (GSH) or similar reducing agents to form arsenite (As^{III}) and glutathione disulfide (GSSG). A methyl group (shown in red) can then be transferred from *S*-adenosylmethionine (SAM) to As^{III} by the As^{III} methyltransferase (AS3MT) to form pentavalent monomethylarsonic acid (MMA^{V}) and *S*-adenosylhomocysteine (SAH). MMA^{V} can then be reduced by thioredoxin (Trx) to trivalent monomethylarsonous acid (MMA^{III}). MMA^{III} can receive a methyl group from SAM, transferred by AS3MT, to form dimethylarsinic acid (DMA^{V}) and SAH.

Cell lines differ in their capacity to methylate arsenic, with human hepatocytes having a relatively high capacity; keratinocytes and bronchial cell lines having a lower capacity; and bladder epithelial cells, lymphoblasts, and fibroblasts having a very limited arsenic methylation capacity (reviewed in [94]). The efficiency of arsenic metabolism also varies considerably between species (reviewed in [95]). Dogs and mice methylate a substantial portion of InAs, with >70% eliminated as DMA (DMA^{III} + DMA^{V}) within days [96-98]. Rats are also very efficient at methylating InAs to DMA [95]. However, DMA accumulates in the red blood cells (RBCs) of rats due to the affinity of DMA^{III} for cysteine 13 in the rat hemoglobin alpha chain [99]. Thus, rats excrete very little DMA [100]. Since this particular cysteine residue is not present in human or mouse hemoglobin, DMA^{III} does not accumulate in the RBCs of these species [100]. Compared with other mammals, rats also excrete a substantial amount of arsenic into bile [95]. In contrast with most animal models, humans are relatively inefficient at fully methylating InAs to DMA and thus excrete a large portion (~10-20%) of total urinary As (uAs) as MMA (MMA^{III} + MMA^{V}) [95]. Given the large differences in arsenic metabolism between species, and the fact that arsenic by itself is not tumorigenic in most rodent models [101], population-based studies have been essential for studying the potential mechanisms of arsenic toxicity.

Within human populations there is substantial inter-individual variation in arsenic metabolism. Thus far, this has been attributed to differences in nutritional status [102, 103] and genetics [104]. There are also substantial differences by sex, with women typically having a greater capacity to methylate arsenic than men, such that they excrete a lower proportion of uAs as MMA and a higher proportion as DMA [105-107]. This difference is most apparent in adults between the ages of 22 and 55 [108] and may be due to sex differences in the OCM pathway [77]. For example, endogenous choline synthesis may be higher among premenopausal women

due to estrogen-induced upregulation of *PEMT* expression ([109] and reviewed in [110]). Studies in mice suggests that inter-individual differences in the microbiome may also contribute to variation in arsenic metabolism [111-113]. However, this has not been studied in human populations.

The efficiency of arsenic metabolism may also vary with the dose of arsenic, as InAs has been shown to inhibit its own methylation *in vitro* [114, 115]. The second methylation step (i.e., the methylation of MMA to DMA) is particularly sensitive to this [114, 115]. Consistent with this, mice exposed to increasing doses of InAs excrete a larger proportion of arsenic as MMA and a lower proportion as DMA [116]. Therefore, individuals exposed to particularly high concentrations of InAs may have a reduced capacity to completely methylate InAs to DMA. However, while there is some supporting evidence of this in human populations [117-119], the findings have not always been consistent [120].

3. Proposed mechanisms of arsenic toxicity

General mechanisms of arsenic toxicity

There are likely multiple mechanisms of arsenic toxicity. Since As^V and phosphate are structurally very similar, one mechanism of action is through the disruption of phosphate-dependent biochemical reactions and anion transporters [121]. Trivalent arsenical species are also highly toxic, because they react with sulfhydryl groups in proteins and thereby inhibit the activity of many enzymes [122]. In this manner, it has been estimated that arsenic inhibits at least 200 different enzymes [122].

Proposed mechanisms of arsenic carcinogenicity

Although arsenic causes skin, bladder, and lung cancers, and has been associated with several other types of cancer, it is not a traditional mutagen [121]. However, several studies have demonstrated that arsenic causes chromosomal aberrations, generates oxidative stress, inhibits DNA repair, and alters cell cycle control and proliferation (reviewed in [94]). There is also increasing evidence that arsenic induces epigenetic dysregulation [123]. Therefore, arsenic may initiate and/or promote the development of cancer through multiple pathways.

4. Arsenic metabolism and toxicity

The relative toxicities of the predominant arsenic metabolites are shown in **Figure 5**. *In vitro*, the trivalent arsenical species, particularly the methylated metabolites, are the most cytotoxic (reviewed in [103]). However, DMA^{III} is highly unstable and thus may only contribute to a very small portion of total arsenic *in vivo* [124]. Among the pentavalent arsenic metabolites, As^{V} is the most cytotoxic, followed by MMA^{V}, then DMA^{V} (reviewed in [103]).

Figure 5. Relative toxicities of predominant arsenic species. The most cytotoxic arsenic metabolites are shown to the left in red shades and the least cytotoxic metabolites are shown to the right in blue shades. The trivalent metabolites, particularly the methylated metabolites (MMA^{III} and DMA^{III}) are the most cytotoxic *in vitro*. However, DMA^{III} (not shown) is highly unstable and may not be present in large quantities *in vivo* [124]. Of the pentavalent metabolites, As^{V} has been shown to be the most toxic, followed by the methylated metabolites (MMA^{V} and DMA^{V}), with DMA^{V} being the least cytotoxic species.

The relative toxicities of the arsenic metabolites also depend on their circulating half-lives. In hamsters, the methylated arsenic metabolites are eliminated very rapidly, with half-lives of 7.4 and 5.6 hours for MMA and DMA, respectively [125]. Although the corresponding half-lives are unknown for humans, DMA is the predominant metabolite observed in urine, followed by MMA, then InAs, providing additional evidence that DMA is the most rapidly eliminated arsenic metabolite (reviewed in [126]). Consistent with this, *As3mt* knockout mice exposed to InAs excrete less DMA and total uAs and retain a higher body burden of arsenic compared with wildtype animals exposed to the same dose [127, 128], indicating that arsenic methylation facilitates urinary arsenic excretion. *As3mt* knockout mice are also more susceptible to arsenic toxicity [129], which further suggests that arsenic methylation is a detoxification process. This is also supported by studies in human populations, which have demonstrated that arsenic-exposed individuals with a higher proportion of MMA (%MMA) in urine have an increased risk of developing adverse health outcomes, including lung [130, 131], bladder [131-135], and skin cancers [136-138]; skin lesions [139-141]; peripheral vascular disease (PVD) [142]; cardiovascular disease [143, 144]; developmental delay [145]; and breast cancer [146], although this has not been observed for hypertension [147, 148] or diabetes [149] (**Figure 6** and **Appendix**, **Table A1**). Many of these studies also examined the proportion of DMA in urine (%DMA) and generally observed inverse relationships with adverse outcomes (**Figure 7** and **Appendix**, **Table A2**). Notably, there were two exceptions to these trends: 1) in contrast to the finding by Huang et al. [150], Melak et al. observed a positive association between %DMA and bladder cancer [131] and 2) Kuo et al. identified a positive association between %DMA and the incidence of diabetes [149]. However, the relationship between %DMA and diabetes has not yet

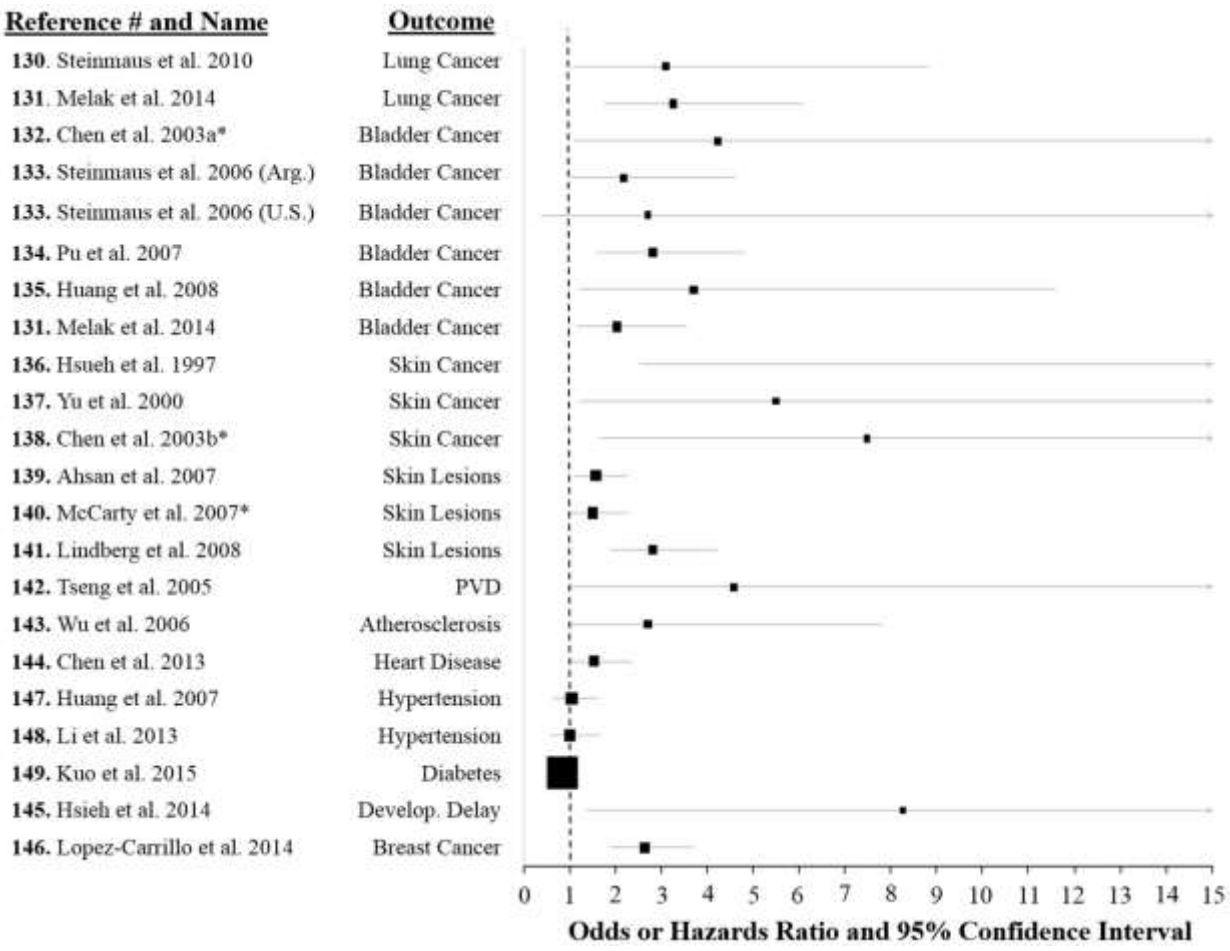

Figure 6. Associations between %MMA in urine and adverse health outcomes. Black boxes represent point estimates for odds or hazards ratios for associations between %MMA and adverse health outcomes. The size of the box is proportional to the precision of the estimate based on the standard error. 95% confidence intervals are represented by gray lines. Arrows indicate that the confidence interval extends beyond the range of the plot. The association is not statistically significant at $P < 0.05$ if the confidence interval crosses the dashed line. Outcomes that have consistently been associated with arsenic exposure are listed first. *MMA:InAs or DMA:MMA (low versus high) was used instead of %MMA. Abbreviations used: Arg., Argentina; Develop. Delay, developmental delay; %MMA, proportion of monomethyl arsenical species (MMA^{III} + MMA^{V}) in urine; DMA:MMA, ratio of dimethyl arsenical species (DMA^{III} + DMA^{V}) to monomethyl arsenical species in urine; MMA:InAs, ratio of MMA to inorganic arsenical species (As^{III} + As^{V}); PVD, peripheral vascular disease; U.S., United States

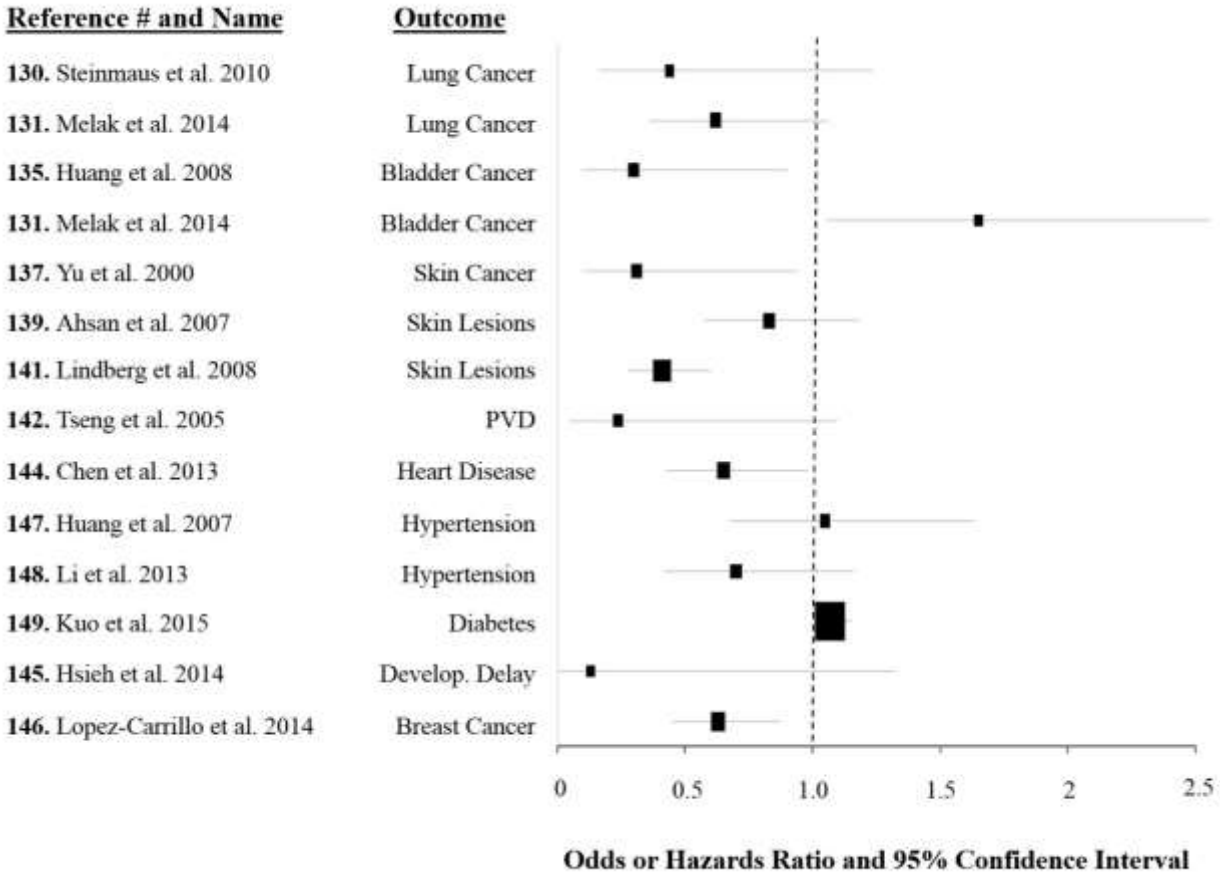

Figure 7. Associations between %DMA in urine and adverse health outcomes. Black boxes represent point estimates for odds or hazards ratios for associations between %DMA and adverse health outcomes. The size of the box is proportional to the precision of the estimate based on the standard error. 95% confidence intervals are represented by gray lines. Arrows indicate that the confidence interval extends beyond the range of the plot. The association is not statistically significant at $P < 0.05$ if the confidence interval crosses the dashed line. Outcomes that have consistently been associated with exposure to arsenic are listed first. Abbreviations used: Develop. Delay, developmental delay; %DMA, proportion of dimethyl arsenical species (DMA^{III} + DMA^{V}) in urine; PVD, peripheral vascular disease

been confirmed in other populations, and whether or not arsenic exposure is causally related to diabetes has been debated [83, 151-153].

5. Influences of one-carbon metabolism on arsenic metabolism and toxicity

The OCM pathway and arsenic metabolism are highly interrelated, particularly since the latter involves two sequential SAM-dependent methylation reactions. There are therefore many possible nutritional influences on the metabolism and toxicity of arsenic.

Experimental studies

Early experimental studies demonstrated that arsenic-exposed rabbits fed a choline- and Met-deficient diet eliminate less arsenic in urine than arsenic-exposed rabbits fed a control diet, primarily due to reductions in DMA excretion [154]. Similarly, mice exposed simultaneously to arsenic and a diet deficient in choline [155] or folate [156] have been shown to excrete less uAs and to incur greater amounts of DNA damage at lower doses of arsenic than mice fed a control diet [155]. Furthermore, mice nullizygous for *Folbp2*, which is involved in folate transport [157], and mice born to mothers nullizygous for *Mthfr* [158] are more susceptible to arsenic-induced embryotoxicity. Choline supplementation also protects against arsenic-induced neural tube defects (NTDs) in chicks [159], and several *in vitro* studies have demonstrated that nutritional methyl donors prevent arsenic-induced DNA damage [160-162], mitochondrial dysfunction [160], alterations in gene expression patterns [163], apoptosis [164], and oxidative stress [165]. Collectively, these studies suggest that nutritional methyl donors protect against arsenic toxicity, and there are likely multiple protective mechanisms.

Observational studies

Epidemiological studies have also provided evidence that the OCM pathway and arsenic metabolism are highly interrelated. Several cross-sectional studies in Bangladesh have observed that nutritional methyl donors, including folate, Met, and choline, are associated with an arsenic metabolite profile indicative of enhanced arsenic methylation capacity (i.e., a lower %MMA and a higher %DMA in urine) and lower concentrations of blood arsenic (bAs), while plasma Hcys has been associated with an arsenic metabolite profile indicative of reduced arsenic methylation capacity (i.e., a higher %MMA and a lower %DMA in urine) and higher concentrations of bAs [166-168]. Studies in Argentinian populations have also observed associations between single nucleotide polymorphisms (SNPs) in OCM genes and arsenic metabolites [169-171]. For example, SNPs which reduce the activity of MTHFR have been associated with a higher %InAs and a lower %DMA in urine [169]. Similarly, SNPs in *CBS* which increase Hcys concentrations are associated with a higher %MMA in urine [170]. Folate deficiency and HHcys are also independent predictors of arsenic-induced skin lesion risk [172], and plasma folate has been inversely associated with bladder cancer risk in an arsenic-exposed population [150], providing additional human evidence that nutritional methyl donors, such as folate, may protect against arsenic toxicity.

Importantly, some of these effects may be population-dependent. For example, studies in the United States have not observed significant associations between dietary folate and uAs metabolites [173] or toenail arsenic concentrations [174]. Since these studies occurred after the United States mandated FA fortification of staple foods, this could be due to the fact that most of these participants were folate sufficient [173, 174]. Another important consideration is that both studies examined dietary folate rather than folate biomarkers. Natural folates are very susceptible

to oxidative degradation, thus dietary folate may not accurately reflect folate status [175]. Similar to the findings in the United States, a study in pregnant Bangladeshi women only observed marginal associations between plasma folate and uAs metabolites [176]. However, these women were taking prenatal supplements, including 400 µg FA/day [176], and were therefore likely to be folate sufficient. Furthermore, the OCM pathway is known to be dramatically altered during pregnancy [177].

Although folate has not been associated with arsenic measures in populations receiving adequate folate, a study in the northeastern United States observed an inverse relationship between dietary cobalamin intake and toenail arsenic concentrations [174]. However, the relationship between cobalamin and arsenic metabolism may be quite complex, as studies in Bangladesh have observed positive associations between plasma cobalamin [178], or dietary cobalamin intake [168], and the %MMA or the ratio of MMA to DMA, respectively, in urine.

Randomized clinical trials

Thus far, two randomized, placebo-controlled trials in Bangladesh have provided the strongest human evidence that folate facilitates the metabolism and excretion of arsenic. The first such trial, which was conducted in folate-deficient adults, demonstrated that 400 µg FA/day significantly reduces the total proportion of uAs excreted as InAs or MMA, increases the total proportion of uAs excreted as DMA [179], and reduces total bAs concentrations [180]. A second trial demonstrated that a higher dose of FA (800 µg FA/day) reduces bAs concentrations in a mixed population of folate-deficient and folate-replete adults, and to a similar extent (12% reduction) as 400 µg FA/day in folate deficient adults (14% reduction) [181].

C. Interplay between arsenic, one-carbon metabolism, and epigenetics

1. Epigenetics

Epigenetics has traditionally been defined as the study of stable and heritable changes in gene expression or cellular phenotypes that occur without changes in Watson-Crick base-pairing of DNA [182]. However, others have proposed a less conservative definition, which includes all chromatin modifications that alter gene activity, irrespective of heritability [183].

Histone proteins

The major unit of chromatin is the nucleosome, which is composed of approximately 147 base pairs of DNA, wrapped 1.65 times around a histone protein octamer [184, 185]. The histone octamer consists of two copies of each of the four core histones: H2A, H2B, H3, and H4; histones H2A and H2B form two heterodimers, while histones H3 and H4 form a tetramer [184, 186] (**Figure 8**). A fifth histone (H1), known as the linker histone, helps to stabilize the nucleosome and facilitates the folding of nucleosomes into higher-order structures [183, 187]. In addition to helping to compact and organize DNA such that it fits inside the nucleus, histone proteins regulate the accessibility of DNA for different cellular processes, such as gene transcription, DNA replication, and DNA repair [188]. Several mechanisms regulate these processes, including the addition of posttranslational modifications to the histone proteins [188], replacement of the canonical histones with histone variants [189], and potentially, proteolytic cleavage of the histone proteins [190] (see Chapter 4 for additional discussion of histone cleavage).

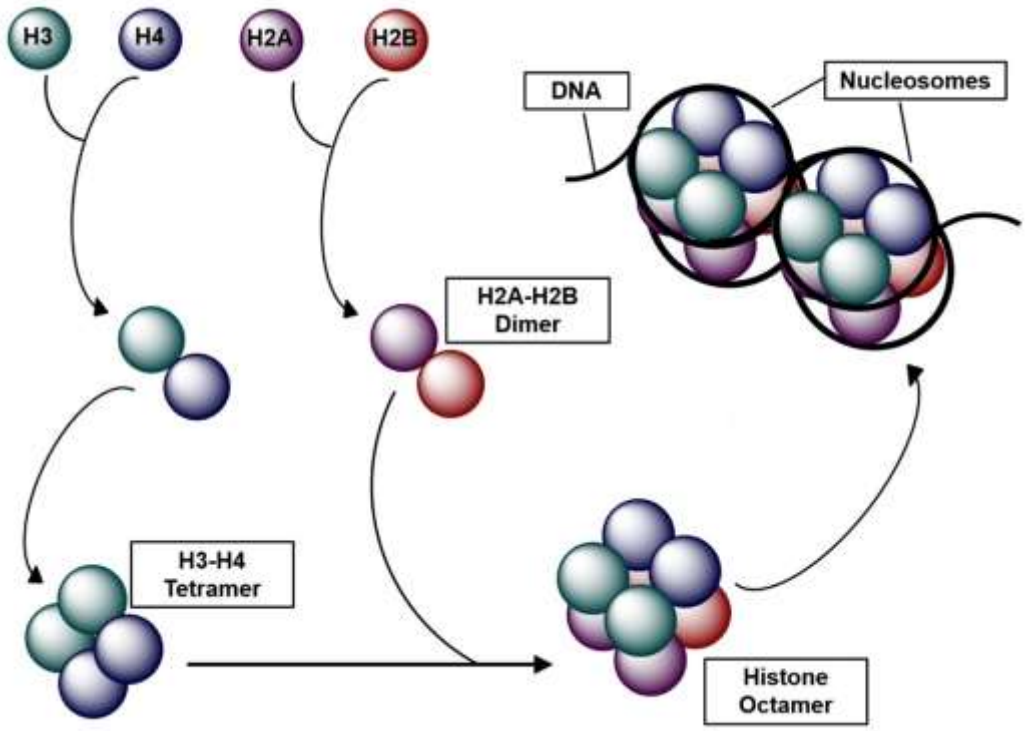

Figure 8. Nucleosome structure. The basic unit of chromatin is the nucleosome, which is composed of 147 base pairs of DNA wrapped around a histone octamer. The histone octamer is comprised of two copies of each of the four core histone proteins: H2A, H2B, H3, and H4. Histones H2A and H2B form two dimers, while two copies of histones H3 and H4 form a tetramer. Figure adapted from Chen et al. 2014 [192].

Histone structure

Histones have two major domains: a globular core and a long, unstructured N-terminal tail (**Figure 9**). Although posttranslational histone modifications (PTHMs) can be added to amino acids in both domains, PTHMs in the N-terminal tails have historically received the most attention. This is largely due to the fact that PTHMs were first identified by Edman degradation, which is limited to measuring the first 20 to 30 amino acids within the N-terminus of a protein

[191]. However, the relatively recent application of mass spectrometry for histone characterization has led to the discovery of numerous PTHMs located within the globular core domains of these proteins, which also play important roles in chromatin regulation and are now receiving more attention [191].

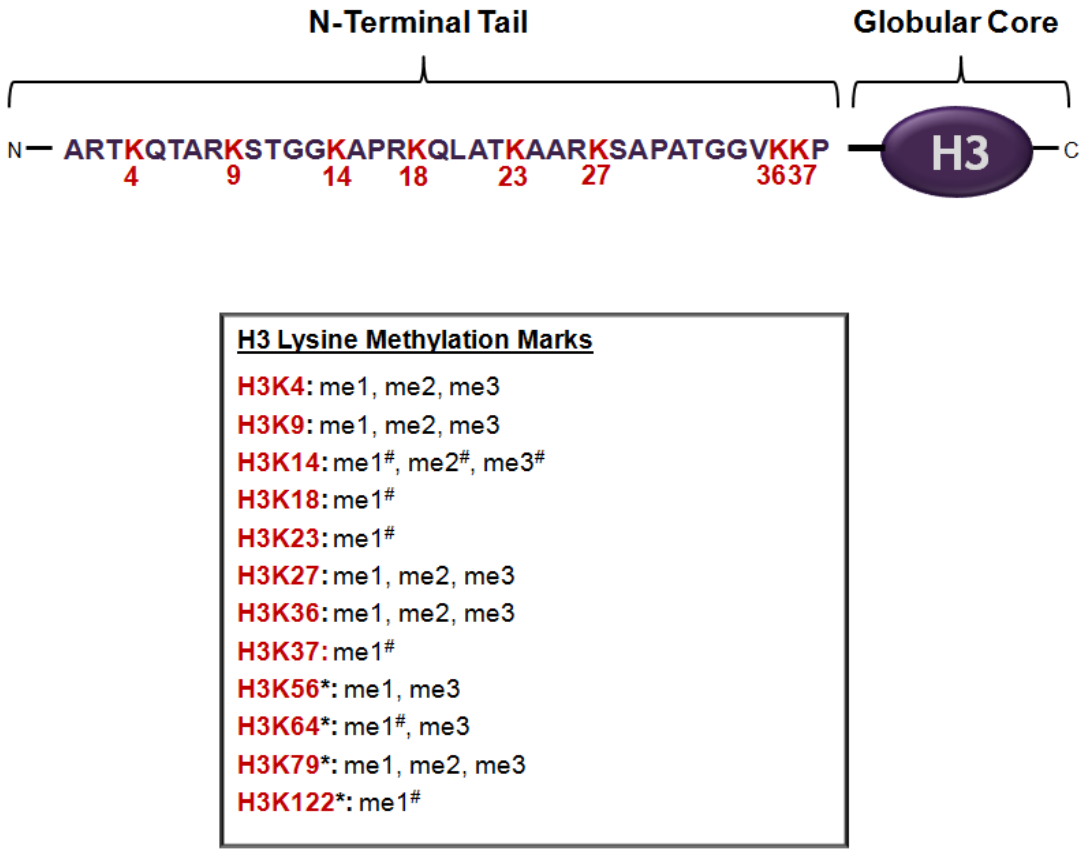

Figure 9. Human histone H3 structure and lysine methylation marks. Histone proteins, including H3, have two major domains: a globular core and an N-terminal tail. Many of the amino acids within each domain can be modified with different moieties. One of the best studied modifications is lysine methylation. Human H3 lysine residues that are known to be methylated are shown in red and are listed in the box above. Most of these lysine residues can be modified with one, two, or three methyl groups (me1, me2, me3, respectively). #Identified but currently no known function. *Located in globular core domain.

PTHM nomenclature

PTHMs are named based on which protein is modified (i.e., H3, H4, H2A, H2B, or H1); the type of amino acid that is modified, indicated by its single letter abbreviation; the position of the modified amino acid in relation to the N-terminus, indicated by a number denoting its location; the type of modification present (e.g., acetylation vs. methylation); and, in the case of methylation, the number of methyl groups present. Of the four core histones, H3 is the most highly modified [193]. Recently, Xu et al. summarized all human H3 PTHMs that have been identified by mass spectrometry [193]. Although 17 different types of PTHMs on more than 30 amino acids have been characterized, the functions of many remain unknown [193]. To date, the best described modifications include the acetylation and methylation of lysine residues. Histone lysine residues can be modified with a single acetyl group (ac) or with one, two, or three methyl groups (me1, me2, and me3, respectively). All known lysine methylation marks identified in human H3 are depicted in Figure 9.

DNA modifications

The best-studied DNA modification is 5-methylcytosine (5-mC), which is established with the transfer of a methyl group from SAM to the 5' position of a cytosine residue (**Figure 10**) [194]. This can be catalyzed by one of several DNA methyltransferases (DNMTs) [194]. DNA methylation generally occurs at cytosine-guanine dinucleotides (CpG sites) and in a symmetrical fashion [195]. In mammals, approximately 70%-80% of CpG sites are methylated [196]. 5-mC is observed at high levels in repetitive elements, which prevents genomic instability, and within gene bodies, where it facilitates transcriptional elongation and prevents spurious transcription [197]. In contrast to the rest of the genome, CpG rich regions called CpG islands

(CGI) tend to be unmethylated, allowing for active transcription of the associated genes [198]. Although definitions for CGIs vary, they are typically classified as regions of at least 200 base pairs in length, with a G+C content >50% and an observed-to-expected CpG ratio >60% [198]. CGIs are present in more than 50% of genes [198]. Methylation in these regions may inhibit transcription directly by obstructing transcription factor binding or indirectly by recruiting chromatin modifying enzymes, which restructure the chromatin such that it is less accessible to the transcriptional machinery [199]. Although gene-specific levels of DNA methylation are cell type specific, genome-wide DNA methylation levels have been shown to be highly correlated between purified cell types within blood and skin lineages (Pearson's correlations typically between 0.96 and 0.99) [200].

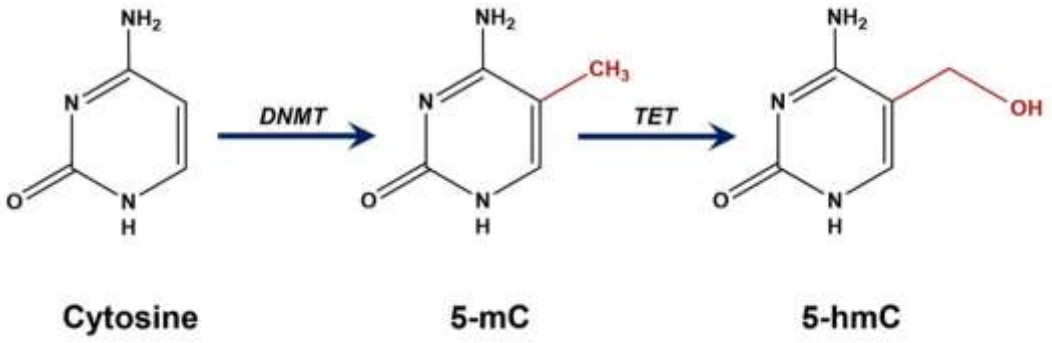

Figure 10. 5-methylcytosine and 5-hydroxymethylcytosine. Cytosine residues within DNA can be modified with different moieties, such as methyl or hydroxymethyl groups (shown in red). 5-methylcytosine (5-mC) is formed when a methyl group is transferred from SAM to the 5' position of a cytosine residue in a reaction that can be catalyzed by one of three DNA methyltransferases (DNMTs). 5-mC can then be oxidized to 5-hydroxymethylcytosine (5-hmC) by one of several enzymes within the ten-eleven translocation methylcytosine dioxygenase (TET) family. 5-hmC can be further oxidized to other cytosine derivatives, which can subsequently be removed by the base excision repair pathway.

Although 5-mC is considered a stable mark, a global loss of 5-mC occurs with aging and during cancer development [201-203]. Originally it was thought that DNA demethylation only occurred through passive mechanisms. However, there is now evidence that 5-mC is also actively demethylated through a pathway that depends on the oxidation of 5-mC to 5-hydroxmethylcytosine (5-hmC) [204]. This is catalyzed by the ten-eleven translocation (Tet) methylcytosine dioxygenase family of enzymes [204] (Figure 9). 5-hmC can be further oxidized to other cytosine derivatives [204], which can be removed by the base excision repair pathway (reviewed in [205]).

In most mammalian tissues, 5-hmC is much less abundant than 5-mC [195]. 5-hmC levels range from 0.03% to 0.17% in most mouse tissues [206]. One exception to this is the central nervous system, where normal 5-hmC levels may be as high as 0.70% [206]. In contrast, global 5-mC levels are close to 4.30% in almost all tissues [206]. Although the biological roles of 5-hmC are not fully understood, this mark has generally been associated with active genes and may be important for epigenetic reprogramming [207].

Euchromatin and heterochromatin

PTHMs and DNA methylation work together to regulate chromatin, which can be classified into two major regions: heterochromatin and euchromatin [208]. Heterochromatin is condensed and is generally transcriptionally inactive, whereas euchromatin is open and mainly contains transcriptionally active genes [208] (**Figure 11**). Each region is dominated by specific epigenetic patterns (Figure 11). Heterochromatin is typically characterized by high levels of 5-mC, low levels of 5-hmC, and repressive PTHMs, such as H4K20me3, H3K9me2/3, and H3K27me3 [184, 188, 209, 210]. In contrast, euchromatin is typically characterized by low

levels of 5-mC, high levels of 5-hmC, and activating PTHMs, such as acetylation at H3K14, H4K16, and H4K18, and methylation at H3K4, H3K36, and H3K79 [184, 188, 209, 210].

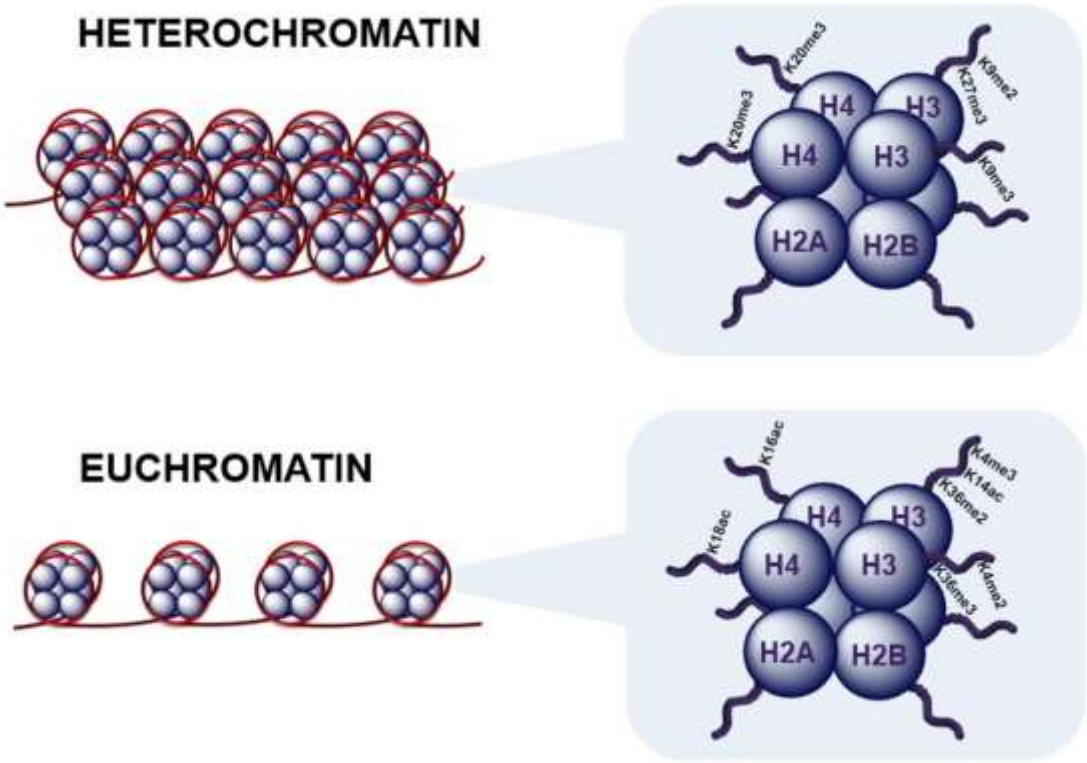

Figure 11. Heterochromatin and euchromatin. Chromatin consists of DNA (shown in red) wrapped around a histone octamer, consisting of two copies of each of the core histone proteins: H2A, H2B, H3, and H4 (shown in purple). Chromatin can be classified broadly into two major domains: heterochromatin (top), which is highly condensed and transcriptionally inactive, and euchromatin (bottom), which is open, accessible, and generally transcriptionally active. Each region is characterized by specific epigenetic modifications. These are depicted in the insets, which convey a zoomed-in perspective of each region of chromatin. Heterochromatin is characterized by repressive PTHMs, such as H3K9me2/3, H3K27me3, and H4K20me3, and high levels of 5-mC (not shown). In contrast, euchromatin is characterized by PTHMs associated with transcriptional activation or elongation, such as H3K4me3, H3K14ac, H3K4me2, H3K36me2, H3K36me3, H3K79me2 (located in H3 core domain), H4K16ac, and H4K18ac, and high levels of 5-hmC (not shown).

PTHMs can alter chromatin structure through both direct and indirect mechanisms. Since acetyl and phosphate groups are negatively charged, the addition of these moieties to histones directly impacts chromatin structure by reducing the affinity of the otherwise positively charged histone proteins for the negatively charged DNA [188]. This leads to a more open chromatin conformation [188]. However, PTHMs can also indirectly alter chromatin structure by recruiting chromatin modifiers, which actively remodel the chromatin to a more open or closed conformation [188].

Relationship between PTHMs and DNA methylation

The relationship between PTHMs and DNA methylation is dynamic, with each type of modification influencing the other. PTHMs have been shown to direct *de novo* DNA methylation patterns during development [211]. For example, methylation at H3K4 blocks the binding of DNMT3L, a recruiter of *de novo* DNMTs [212]. In turn, DNA methylation can act as a template for the re-establishment of PTHMs after DNA has been replicated [211]. However, PTHMs are generally thought to be more labile than DNA methylation [211]. Therefore, PTHMs may be more easily influenced by environmental exposures and may thus mediate environmentally-induced alterations in DNA methylation.

Regulation of histone lysine methylation

Histone lysine methylation marks are regulated by histone lysine demethylases (KDMs) and SAM-dependent lysine histone methyltransferases (KHMTs). KDMs can be classified into two major groups. The majority fall under the Jumonji C family, which can remove methyl groups from mono-, di-, and tri-methylated substrates (reviewed in [213]). In contrast, the LSD family of demethylases can only demethylate mono- and di-methylated substrates [213]. KHMTs

are also classified into two major groups based on their catalytic domains. The majority of KHMTs have a highly conserved Su(var)3-9, Enhancer of Zeste [E(Z)], and Trithorax (trx) domain [214]. The only exception is DOT1L, which catalyzes the mono- di- and tri-methylation of H3K79, but is structurally more similar to arginine methyltransferases [214].

2. Dysregulation of epigenetic modifications in human diseases

DNA methylation and human diseases

Distinct alterations in DNA methylation patterns have been observed in numerous cancers and in several non-cancer health outcomes. Typically, a simultaneous loss of global, but gain of gene-specific, DNA methylation is observed during cancer development (reviewed in [215]). This can lead to genomic instability and the silencing of tumor suppressor genes, respectively [215]. Pre-diagnostic measures of global and genome-wide leukocyte DNA methylation patterns have been associated with increased risks for developing several adverse outcomes, including breast [216, 217] and gastric [218] cancers in women, renal cell carcinoma [219] and ischemic heart disease [220] in men, and arsenic-induced skin lesions [172].

PTHMs and human diseases

Experimental studies have demonstrated that PTHMs are critical for normal cellular processes. For example, DNA repair mechanisms are regulated by several PTHMs, including H3K36me3 [221-223], H3K36me2 [224], and H3K79me2 [214]. H3K79me2 is also required for normal cell cycle control, cardiac development, and hematopoiesis (reviewed in [214]). The dysregulation of these, and other, PTHMs has therefore been implicated in the development of human diseases, particularly cancers. For example, an aberrant global increase in H3K36me2

leads to the overexpression of genes involved in oncogenic programming [225]. Similarly, a global aberrant increase in H3K79me2 plays a key role in the development of MLL-fusion leukemia [214]. In contrast, global reductions in H4K16ac and H4K20me3 are characteristic of most cancer types [226]. Global PTHM patterns have also been used successfully to predict the prognosis of many types of cancer, including lung, prostate, breast, gastric, esophageal, kidney, liver, colorectal, and pancreatic cancers, as well as gliomas and hematological malignancies ([227-230] and reviewed in [231]).

There is also evidence that PTHMs are dysregulated in non-cancer outcomes. For example, human NTDs are characterized by a global loss of H3K79me2 [232], and alterations in H3 phosphorylation and acetylation have been observed in postmortem brain tissue samples from individuals with Alzheimer's disease [233] and neuropsychiatric disorders [233, 234].

Epigenetic therapeutics

Since epigenetic modifications are reversible, they are targets of many potential therapeutics. Currently, two DNMT inhibitors and three histone deacetylase inhibitors are approved by the Food and Drug Administration for the treatment of hematological malignancies [183]. Several other epigenetic therapeutics are in preclinical or early clinical trials for the treatment of both hematological malignancies and solid tumors, and many of these newer drugs target KDMs and KHMTs [183]. However, it is important to note that in many cases the effects of epigenetic therapies may be non-specific, as many histone-modifying enzymes also have non-histone targets. One notable exception to this is DOT1L, which exclusively methylates H3K79 and is a promising therapeutic target for MLL-fusion leukemia [235, 236].

3. Arsenic and epigenetics

Arsenic and DNA methylation

Consistently, across *in vitro* and animal studies, arsenic has been shown to reduce global levels of DNA methylation (reviewed in [123]). In contrast, positive associations between arsenic exposure and global DNA methylation have been observed in human populations [237-240]. There may be several reasons for these discrepancies: 1) experimental studies often use very high doses of arsenic, which are not relevant to human exposures, 2) the durations of exposure used in experimental studies are much shorter than the chronic exposures experienced by most human populations, 3) *in vitro* studies typically employ cancer/transformed cell lines, and 4) arsenic metabolism and susceptibility to arsenic toxicity differ substantially between humans and animal models. Human populations are also heterogeneous with respect to nutritional status, which can have profound influences on epigenetic marks (described in more detail below). Additionally, there has been a historical bias in the use of male animals and male-derived cell lines for biomedical research, despite important sex differences [241]. Therefore, differences in nutritional status and sex are additional factors which may contribute to inconsistencies across studies. Several population-based studies have begun to tease apart the important interactions between arsenic, nutrition, and sex in relation to DNA methylation.

Interactions with nutritional status and sex

Folate nutritional status has been shown to modify the relationship between arsenic exposure and global DNA methylation. One study in Bangladeshi adults observed a positive association between arsenic exposure and global levels of leukocyte DNA methylation, but only among those who were folate sufficient (plasma folate >9 nmol/L) [237]. Similarly, in a study of

elderly men, arsenic exposure was found to be positively associated with Alu methylation (an indicator of global DNA methylation), but only in individuals with plasma folate concentrations below the median [240]; importantly, since this study took place in the United States after FA fortification occurred, individuals in the low folate stratum were comparable to the folate sufficient stratum in Bangladesh. Thus, the findings from these two studies are consistent.

Sex may also modify the effect of arsenic on DNA methylation. In Bangladesh, prenatal arsenic exposure has been positively associated with global levels of cord blood DNA methylation among males, but negatively among females [242]. Another study in Bangladesh observed that prenatal arsenic exposure was associated with altered cord blood DNA methylation levels in both boys and girls, but the effects were more pronounced among boys [243]. The majority of studies which have examined the relationship between arsenic exposure and global DNA methylation have relied on bisulfite-conversion techniques, which cannot distinguish between 5-mC and 5-hmC (reviewed in [244]). However, in a recent study, which was able to discriminate between these two marks using an LC-MS/MS assay, arsenic exposure was found to be positively associated with both 5-hmC and 5-mC among men, but was inversely associated with 5-hmC and was not associated with 5-mC among women [245]. These findings were observed in two separate study samples [245]. Thus, sex appears to modify the effects of arsenic exposure on both 5-mC and 5-hmC.

Statistical power considerations often preclude the examination of three-way interactions between sex, nutritional status, and arsenic exposure in human populations. However, one study in mice observed that simultaneous exposure to arsenic and a methyl deficient diet (MDD) reduced global levels of DNA methylation in the livers of male animals, but increased global

DNA methylation in the livers of female animals [246]. Therefore, arsenic may interact with both nutrition and sex to influence DNA methylation patterns.

Arsenic and PTHMs

There is increasing evidence that arsenic also alters global levels of PTHMs. While the majority of these studies have been conducted *in vitro* [247-265], there are a few supporting studies in rodents [266, 267] and human populations [268, 269] (**Table 1**). Collectively, these studies provide evidence that arsenic induces global dysregulation of many different PTHMs. However, there are some discrepancies across studies, which may be attributed to differences in the particular tissues/cell lines examined; the durations, doses, and forms of arsenic used; the sex of the animals or cell lines utilized; and, potentially, cleavage of histone proteins, which can affect the measurement of downstream PTHMs (See Chapter 4). Details of each study are summarized in Table 1. Similar to studies on arsenic and DNA methylation, there is increasing evidence that arsenic affects certain PTHMs differentially by sex [267, 269].

4. One-carbon metabolism and epigenetics

One-carbon metabolism and DNA methylation

DNMTs are SAM-dependent enzymes. Therefore, the majority of experimental studies have demonstrated that global levels of DNA methylation are reduced when methyl donors or related cofactors are limiting [270-282] and are increased as a result of methyl donor supplementation [280, 283], although there have been a few exceptions [284-290]. Methyl donors may also counteract epigenetic dysregulation caused by environmental toxicants. For example, Dolinoy et al. observed that *in utero* exposure to bisphenol A reduces DNA

methylation, but this could be prevented by maternal supplementation with a combination of FA, cobalamin, choline, and betaine [291].

Since the methyl deficient conditions used in most animal studies are generally not relevant to human populations, epidemiological studies have been essential for understanding how nutritional methyl donors may influence DNA methylation. Several observational studies have observed inverse relationships between plasma Hcys or SAH concentrations and global DNA methylation levels [293, 294]. Additionally, RBC folate has been positively correlated with DNA methylation, although only in individuals with the T/T *MTHFR* SNP, which reduces MTHFR activity [295, 296]; this SNP has also been independently associated with lower global leukocyte DNA methylation levels [297]. Studies in adult women have also observed that global DNA methylation levels are reduced in response to folate depletion [298, 299], and these effects may be reversible [298]. Additionally, two randomized trials in adults have demonstrated that FA supplementation, at doses ranging from 100 to 4000 μg/day, either alone or in combination with cobalamin (500 μg/day), for durations ranging from 1 month to up to 2 years, alters DNA methylation levels in white blood cells [300, 301].

In contrast with most of the findings for folate, one study observed that maternal choline intake during early pregnancy was inversely associated with cord blood DNA methylation, although this was only observed among boys [302]. Thus, other factors, such as sex and the timing of methyl donor supplementation, may be important modifiers of these relationships.

Table 1. Summary of studies examining the effects of arsenic on global levels of PTHMs

Reference	Cell Line, Mouse Strain, or Population	Sex[1]	Exposure; Doses[2]	Duration	PTHMs examined	Findings
Cell Culture						
[247] Arrigo (1983)	Kc 161 (Drosophila, embryonic)	?	$NaAsO_2$ (As^{III}); 50 µM	4 h	H3, H4, H2A, H2B methylation and acetylation	Methylation: ↓H3, H4, ↑H2B Acetylation: ↓H3, H4, H2A, H2B
[248] Desrosiers and Tanguay (1986)	Schneider and Kc III cells (Drosophila, embryonic)	?	$NaAsO_2$ (As^{III}); 50 µM	4 h	H3, H4, and H2B methylation and acetylation	Methylation: ↓H3, H4, ↑H2B Acetylation: ↓H3, H4, H2B
[265] Cobo et al. (1995)	CHO (Chinese hamster, ovary)	F	$NaAsO_2$ (As^{III}); 10 µM	2 h	H1, H2A, H3, H4 phosphorylation	Phosphorylation: ↓H1 and H3 No effects on H2A or H4
[257] Perkins et al. (2000)	HL-60 (human, APL)/ K562 (human, CML)	F	As_2O_3 (As^{III}); 1, 2 µM/2 µM	7 d/24 h	H3 and H4 acetylation	↑Acetylation for all doses and durations
[249] Li et al. (2002)	NB4 (human, APL)	F	As_2O_3 (As^{III}); 0.4, 0.8, 1.6 µM	24 h	H3S10phK14ac, H3S10ph, H3K14ac, H3K9acK14ac	0.8 and 1.6 µM ↑H3S10ph and H3S10phK14ac No effect on H3K14ac or H3K9acK14ac
[292] Kannan-Thulasiraman et al. (2006)	KT-1 (human, CML)/ NB4 (human, APL)	M/F	As_2O_3 (As^{III}); 2 µM	20 min	H3S10ph	↑H3S10ph
[250] Ramirez et al. (2008)	HepG2 (human, liver cancer)	M	$NaAsO_2$ (As^{III}); 7.5 µM	24 h	H3K4me2, me3, H3K9ac, H3K9me2, me3, H3K27me3, H4K20me3	↑H3K9ac No effects on methylation marks
[251] Zhou et al. (2008)	A549 (human, lung cancer)/BEAS2B (human, healthy lung, SV40-transformed)	M/M	$NaAsO_2$ (As^{III}); 2.5, 5 µM/1, 2 µM	24 h	H3K4me, me2, me3, H3K9me,me2, me3 H3K27me3, H3K36me2, me3/ H3K9me2	↑H3K9me2,me3, no effect on H3K9me, ↑H3K4me2, me3, ↓H3K4me, ↓H3K27me3, ↑H3K36me3, ↓H3K36me2/↑H3K9me2
[252] Zhou et al. (2009)	A549 (human, lung cancer)	M	$NaAsO_2$ (As^{III}); 1, 5 µM	24 h	H3K4me, me2, me3	↑H3K4me2, me3 ↓H3K4me
[260] Jo et al. (2009)	UROTsa (human, healthy urothelium, SV40-transformed)	F	$NaAsO_2$ (As^{III}); 3 µM $MMA^{III}O$ (MMA^{III}); 1 µM	7 d	H4K16ac	↓H4K16ac

[262] Suzuki et al. (2009)	HepG2 (human, liver cancer)	M	$NaAsO_2$ (As^{III}); 60 µM C_2H_7AsI (DMA^{III}); 0.5 µM	0.5, 1.5, 3, 5, 7 h	H3S10ph	↑H3S10ph
[253] Chu et al. (2011)	UROTsa (human, healthy urothelium, SV40-transformed)	F	$NaAsO_2$ (As^{III}); 1 nM, 3 and 10 µM/ $MMA^{III}O$ (MMA^{III}); 0.3, 1, 3 µM	24 h, 7 d	H3 and H4 acetylation	↓H4K16ac, ↓H3 acetylation
[259] Treas et al. (2012)	RWPE1 (human, healthy prostate)	M	$NaAsO_2$ (As^{III}); 100 pg/mL +/- E2, 100 ng/mL +/- E2	6 months	H3ac, H3K4me3	↑H3ac with As^{III} or E2 alone, even greater ↑ for As^{III} + E2 (100 ng/mL) ↓H3ac for combination of As^{III} + E2 (100 pg/mL) ↑H3K4me3 for combination of As^{III} + E2 (100 ng/mL)
[261] Kim et al. (2012)	3T3 cells (BALB/c mouse, embryo fibroblasts)	F	As_2O_3 (As^{III}); 0.5 µM)	2, 4 wk	H3K27me3	↑H3K27me3
[263] Suzuki et al. (2013)	HepG2 (human, liver cancer)	M	$NaAsO_2$ (As^{III}); 50 µM	0.5, 1, 2, 5 h	H3S10ph	↑H3S10ph
[264] Ge et al. (2013)	UROTsa (human, healthy urothelium, SV40-transformed)	F	CH_3AsI_2 (MMA^{III}); 50 nM	12 wk	Acetylation of H4K5, H4K8, H4K12, and H4K16	↓H4K12ac and H4K16ac No effects on H4K5ac or H4K8ac
[254] Herbert et al. (2014)	Primary human neonatal keratinocytes	?	Arsenic source unspecified (As^{III}); 0.5 µM	1, 12, 24, 48 d	H4K16ac	↑H4K16ac for all durations
[255] Liu et al. (2015)	HeLa (human, cervical cancer)/HEK293T (human, embryonic kidney)	F/F	As_2O_3 (As^{III}); 0.2-0.8 µM	24, 48, 72 h	H4K5ac, H4K8ac, H4K12ac, H4K16ac for both cell lines	↓H4K16ac in both cell lines No changes in other PTHMs
[256] Rahman et al. (2015)	HEK293T (human, embryonic kidney)/UROtsa (human, healthy urothelium, SV40-transformed)	F/F	As_2O_3 (As^{III}); 1, 5 µM As_2O_3 (As^{III}); 0.5, 2.5 µM	72 h 3 h	Acetylation of H3K9, H4K12, and H4K16	↓H3K9ac (UROTsa only after 72 h, both doses) No effect at shorter duration or for other PTHMs
[258] Ray et al. (2015)	HaCaT (human, keratinocytes from healthy skin, SV40-transformed)	M	$NaAsO_2$ (As^{III}); 0-25 µM/10 µM	8 h/0-24h	H3S10ph	↑H3S10ph in dose- and time-dependent manner

Rodent						
[266] Cronican et al. 2013	C57BL6/J (brain, cortex and hippocampus)	Both, combined	$NaAsO_2$ (As^{III}); 100 µg/L	(1 wk before conception until birth)	H3K9ac	↓H3K9ac
[267] Tyler et al. 2015	C57BL/6 (brain, dentate gyrus and frontal cortex)	Both, separate	Na_3AsO_4 (As^V); 50 µg/L	(10 d prior to pregnancy – weaning)	H3K4me3, H3K9ac, H3K9me3	M dentate gyrus: ↑H3K4me3 and ↑H3K9ac F dentate gyrus: ↓H3K4me3 and ↓H3K9ac M frontal cortex: ↑H3K4me3 and ↓H3K9ac F frontal cortex: No change in H3K4me3 or H3K9ac No effects on H3K9me3 in either brain region in either sex
Human						
[268] Cantone et al. 2011	Adults, occupationally exposed via inhalation, Italy (PBLs) (n = 63)	M	Arsenic in particulate matter (0.01 – 0.31 µg/m^3)	Chronic (y)	H3K4me2, H3K9ac	↑H3K4me2
[269] Chervona et al. 2012	Adults, exposed via contaminated drinking water, Bangladesh (PBMCs) (n = 40, 50% male)	Both, separate	Water As (primarily As^{III}) (50 – 500 µg/L)	Chronic (y)	H3K4me3, H3K9ac, H3K9me2, H3K18ac, H3K27ac, H3K27me3	Whole sample: ↑H3K9me2, ↓H3K9ac. M: ↓H3K4me3 and H3K27me3. ↑H3K27ac. F: ↑H3K4me3 and H3K27me3. ↓H3K27ac

Abbreviations used: As^{III}, arsenite; As^V, arsenate; As_2O_3, arsenic trioxide; APL, acute promyelocytic leukemia; C_2H_7AsI, dimethylarsine iodide; CH_3AsI_2, diiodomethylarsine; CML, chronic myelogenous leukemia; d, days; E2, estradiol; F, female; h, hours; M, male; $NaAsO_2$, sodium arsenite; Na_3AsO_4; sodium arsenate; PBLs, peripheral blood leukocytes; PBMCs, peripheral blood mononuclear cells; PTHM, posttranslational histone modification; wk, week; y, years

[1]With respect to cell culture studies, sex refers to the biological sex of the animal or patient from which the cell line was derived
[2]Units are listed as they were reported in the original reference.

One-carbon metabolism and PTHMs

Similar to DNMTs, KHMTs are SAM-dependent enzymes (**Figure 12**). Thus, several experimental studies have also demonstrated that supplementation with choline or folate increases global levels of histone methylation marks [303, 304], while deficiencies in methyl donors typically reduce them [305-307]. However, the effects of nutritional methyl donors on PTHMs may be more complex than their effects on DNA methylation for several reasons. First, there is evidence that PTHMs have differing sensitivities to alterations in methyl donors [304, 306]. It has been hypothesized that this may be due to the fact that KHMTs likely have differing binding affinities for SAM [304]. The effects of nutritional methyl donors on PTHMs may also be complicated by the fact that histone lysine residues can be mono-, di-, or tri-methylated. These methylation states are mutually exclusive. Therefore, a decrease in one methylation state may necessitate the increase of another [308]. There is also substantial cross-talk between PTHMs. Thus, the presence of a particular modification at one amino acid may prevent or promote the addition of a modification at another amino acid [308]. For example, methylation at H3K4 precludes methylation at H3K9, and vice versa [308]. Therefore, it may not be surprising that methyl donor depletion has also been shown to induce higher levels of some PTHMs [303, 309].

Although folate is an important methyl donor, it is also an acceptor and carrier of one-carbon groups. Therefore, folate may have dual roles in regulating histone methylation marks. Two recent studies by the same group observed that folate, in the form of THF, binds to LSD1, a histone demethylase, and likely accepts the one-carbon group as it is removed from the histone protein; this may occur to prevent the formation of formaldehyde during oxidative histone demethylation, which could otherwise cause cross-linking at the enzyme's active site [310, 311].

Consistent with this hypothesis, the same group demonstrated that H3K4me2, a target of LSD1, was increased globally in the livers of male mice fed a folate deficient diet [312]. Thus, the effects of folate on histone methylation may be even more complex than those of other nutritional methyl donors.

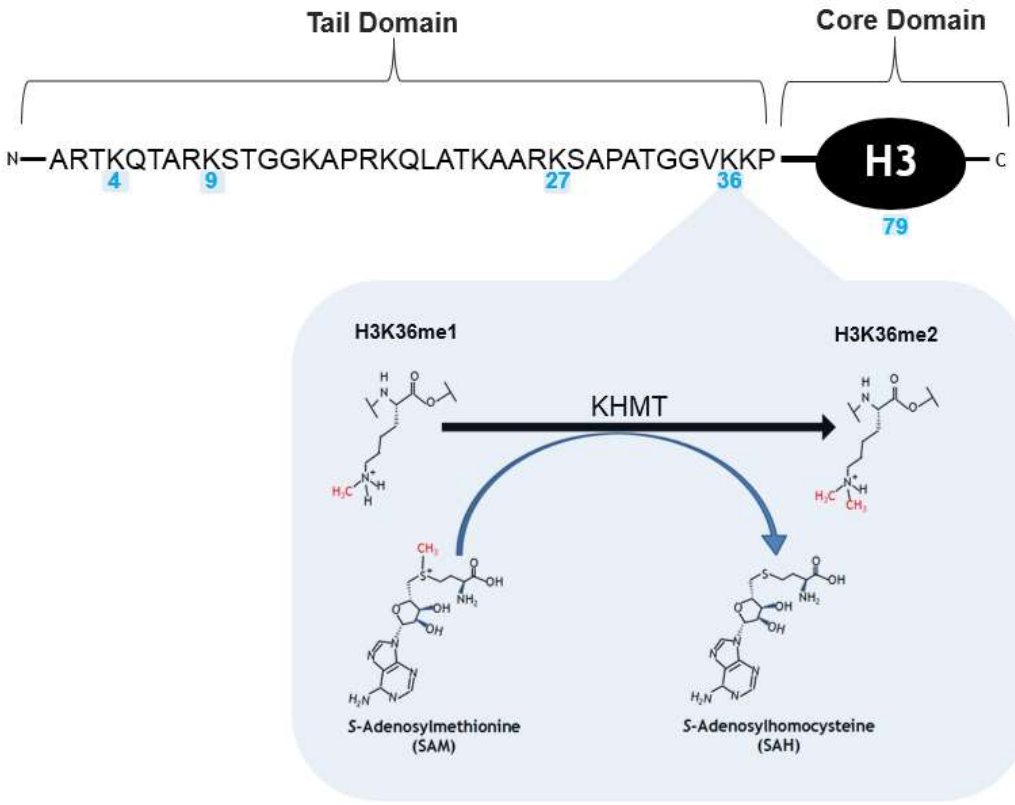

Figure 12. SAM-dependent methylation of histone H3. Commonly examined H3 lysine residues are indicated in light blue. Most histone lysine residues can receive one, two, or three methyl groups. Lysine histone methyltransferases (KHMTs), which depend on methyl donations from *S*-adenosylmethionine (SAM), catalyze these reactions. Once a KHMT has transferred a methyl group from SAM to the histone substrate, *S*-adenosylhomocysteine (SAH) is formed. SAH can inhibit subsequent methylation reactions. The inset depicts the transfer of a methyl group (shown in red) from SAM to the mono-methyl form of H3K36 (H3K36me1), resulting in the di-methyl form of H3K36 (H3K36me2) and SAH.

The effects of OCM indices on PTHMs have been largely understudied in human populations. One very small case-control study in China (n = 8) characterized global PTHM patterns in human NTDs using mass spectrometry, and observed that H3K79me2 levels were much lower in fetal brain tissue from NTD cases compared with healthy controls matched on gestational age and sex [232]. Since folate deficiency is an established risk factor for NTDs, these findings provide indirect evidence that folate deficiency reduces global H3K79me2 levels [232]. This group also confirmed that folate deficient conditions reduce H3K79me2 levels *in vitro* [232]. A second study in the United States by Piyathilake et al. examined the influence of FA fortification on global H3K9me2 levels in women with cervical intraepithelial neoplasia (CIN) [313]. They compared H3K9me2 levels in CIN tissue collected from participants recruited pre- vs. post-FA fortification, and found that H3K9me2 levels were higher in the tissue collected post-fortification, after adjusting for age and race. Piyathilake et al. therefore concluded that FA increased global H3K9me2 levels [313]. However, this study had several important limitations: 1) there were likely other factors which changed during the FA fortification period, or which differed between the two groups of participants, which may have confounded the relationship between FA fortification and H3K9me2, 2) CIN tissue collected pre-FA fortification had been stored for a longer duration than CIN tissue collected post-fortification, which could potentially affect H3K9me2 measures differentially by group, and 3) individual-level folate measures were not available for the participants, thus it is unclear if folate status actually differed between the two groups. Therefore, while there is some preliminary evidence from human populations that folate influences PTHMs, additional epidemiological studies are needed.

D. Summary and rationale

As outlined in sections B and C, there is growing evidence that nutrients involved in the OCM pathway may influence arsenic toxicity through effects on both arsenic metabolism and epigenetics. Since the metabolism of arsenic involves two sequential SAM-dependent methylation reactions, nutrients involved in the OCM pathway, particularly folate, facilitate the methylation and urinary excretion of arsenic. However, the relationships between SAM and arsenic methylation, and the potential modifying effects of nutrients in the OCM pathway, have not been evaluated in human populations. In experimental models, SAM has also been shown to influence epigenetic modifications that are dependent on methylation reactions, such as DNA and histone methylation. These modifications are dysregulated in adverse health outcomes, such as cancers, and are altered by arsenic and nutritional methyl donors in experimental settings. However, given important differences between experimental models and human populations, there is a need for supporting epidemiological studies. In particular, there is a dearth of information on the influences of arsenic and OCM indices on PTHMs. Since susceptibility to arsenic toxicity differs between men and women, and since previous studies have observed that arsenic influences epigenetic modifications in a sex-dependent manner, a better understanding of potential differences by sex is also critical. The goal of this dissertation is to address these gaps in the literature, using data from three epidemiological studies of arsenic-exposed Bangladeshi men and women.

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CHAPTER TWO APPENDIX

Table A1. Associations between %MMA in urine and adverse health outcomes

Study	Outcome	Predictor	Cutoffs	Design	# Cases	Country	Subgroup	OR/HR (95% CI)
[1] Steinmaus et al. 2010	Lung Cancer	%MMA	>17.2%	Case Control	45	Argentina	NA	3.09 (1.08-8.81)
[2] Melak et al. 2014	Lung/ Bladder Cancer	%MMA	>12.5%	Case Control	94/117	Chile	NA	3.26 (1.76-6.04)/ 2.02 (1.15-3.54)
[3] Chen et al. 2003a	Bladder Cancer	Low vs. high DMA:MMA	≤4.8	Case Control	49	Taiwan	CAE >12 mg/L-yr	4.23 (1.12-16.01)
[4] Steinmaus et al. 2006	Bladder Cancer	%MMA	>16.7%/ >14.8%	Case Control	114/23	Argentina/U.S.	Smokers/ Arsenic >100 µg/day	2.17 (1.02-4.63]/ 2.7 (0.4-18.6)
[5] Pu et al. 2007	Bladder Cancer	%MMA	>9.2%	Case Control	177	Taiwan	NA	2.8 (1.6-4.8)
[6] Huang et al. 2008	Bladder Cancer	%MMA	>11.4%	Case Control	37	Taiwan	CAE >20 mg/L-yr	3.7 (1.2-11.6)
[7] Hsueh et al. 1997	Skin Cancer	%MMA	>26.7%	Case Control	33	Taiwan	CAE >20 mg/L-yr	23.9 (2.6-225.2)
[8] Yu et al. 2000	Skin Cancer	%MMA	>15.5%	Case Control	26	Taiwan	NA	5.5 (1.2-24.8)
[9] Chen et al. 2003b	Skin Cancer	DMA:MMA (Low vs. high)	≤5	Case Control	76	Taiwan	CAE >15 mg/L-yr	7.5 (1.7-34.0)
[10] Ahsan et al. 2007	Skin Lesions	%MMA	>16.4%	Case Control	594	Bangladesh	NA	1.6 (1.1-2.3)
[11] McCarty et al. 2007	Skin Lesions	MMA:InAs	>10	Case Control	600	Bangladesh	NA	1.5 (1.0-2.3)
[12] Lindberg et al. 2008	Skin Lesions	%MMA	>12%	Case Control	504	Bangladesh	NA	2.8 (1.9-4.2)
[13] Tseng et al. 2005	PVD	%MMA	>11.4%	Case Control	54	Taiwan	CAE >0 mg/L-yr	4.6 (1.0-20.6)
[14] Wu et al. 2006	Atherosclerosis	%MMA	>13.4%	Case Control	163	Taiwan	CAE >1.7/mg/L-yr	2.7 (1.0-7.8)
[15] Chen et al. 2013	Heart Disease	%MMA	Highest tertile	Prospective	211	Bangladesh	NA	2.3 (1.0-1.5)
[16] Huang et al. 2007	Hypertension	%MMA	>15.6%	Case Control	372	Taiwan	NA	1.04 (0.66-1.62)
[17] Li et al. 2013	Hypertension	%MMA	>16.4%	Case Control	168	China	NA	0.99 (0.59-1.66)
[18] Kuo et al. 2015	Diabetes	%MMA	Per 5% increase	Prospective	396	U.S. (Native American)	NA	0.84 (0.76-0.94)

[19] Hsieh et al. 2014	Develop. Delay	%MMA	>1.55%	Case Control	63	Taiwan	NA	8.3 (1.4-49.7)
[20] Lopez-Carrillo et al. 2014	Breast Cancer	%MMA	>13.3%	Case Control	1016	Mexico	NA	2.63 (1.89-3.66)

Abbreviations used: CAE, cumulative arsenic exposure; Develop. Delay, developmental delay; DMA:MMA, ratio of dimethyl to monomethyl arsenical species; HR, hazards ratio; %MMA, proportion of monomethyl arsenical species in urine; MMA:InAs, ratio of monomethyl to inorganic arsenical species; OR, odds ratio; PVD, peripheral vascular disease; U.S., United States

Table A2. Associations between %DMA in urine and adverse health outcomes

Study	Outcome	Predictor	Cutoffs	Design	# Cases	Country	Subgroup	OR/HR (95% CI)
[1] Steinmaus et al. 2010	Lung Cancer	%DMA	>74%	Case Control	45	Argentina	NA	0.44 (0.16-1.23)
[2] Melak et al. 2014	Lung/ Bladder Cancer	%DMA	≥83.9%	Case Control	94/117	Chile	NA	0.62 (0.36-1.06)/ 1.65 (1.06-2.56)
[6] Huang et al. 2008	Bladder Cancer	%DMA	≥85.8%	Case Control	37	Taiwan	NA	0.3 (0.1-0.9)
[8] Yu et al. 2000	Skin Cancer	%DMA	>72.2%	Case Control	26	Taiwan	NA	0.3 (0.1-0.9)
[10] Ahsan et al. 2007	Skin Lesions	%DMA	≥76.1	Case Control	594	Bangladesh	NA	0.83 (0.58-1.18)
[12] Lindberg et al. 2008	Skin Lesions	%DMA	>82%	Case Control	504	Bangladesh	NA	0.41 (0.28-0.60)
[13] Tseng et al. 2005	PVD	%DMA	>81.01%	Case Control	54	Taiwan	CAE >0 mg/L-yr	0.24 (0.05-1.10)
[15] Chen et al. 2013	Heart Disease	%DMA	≥75.6%	Prospective	211	Bangladesh	NA	0.65 (0.42-0.98)
[16] Huang et al. 2007	Hypertension	%DMA	≥85.3	Case Control	372	Taiwan	NA	1.05 (0.68-1.63)
[17] Li et al. 2013	Hypertension	%DMA	>79.1%	Case Control	168	China	NA	0.70 (0.42, 1.16)
[18] Kuo et al. 2015	Diabetes	%DMA	Per 5% increase	Prospective	396	U.S. (Native American)	NA	1.07 (1.00-1.15)
[19] Hsieh et al. 2014	Develop. Delay	%DMA	>96%	Case Control	63	Taiwan	NA	0.13 (0.01-1.32)
[20] Lopez-Carrillo et al. 2014	Breast Cancer	%DMA	>85%	Case Control	1016	Mexico	NA	0.63 (0.45-0.87)

Abbreviations used: CAE, cumulative arsenic exposure; Develop. Delay, developmental delay; %DMA, proportion of dimethyl arsenical species in urine; HR, hazards ratio; OR, odds ratio; PVD, peripheral vascular disease; U.S., United States

CHAPTER TWO APPENDIX REFERENCES

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CHAPTER THREE

Folate and cobalamin modify associations between *S*-adenosylmethionine and methylated arsenic metabolites in arsenic-exposed, Bangladeshi adults

Caitlin G. Howe[1] Megan M. Niedzwiecki[1], Megan N. Hall[2], Xinhua Liu[3], Vesna Ilievski[1], Vesna Slavkovich[1], Shafiul Alam[4], Abu B. Siddique[4], Joseph H. Graziano[1], and Mary V. Gamble[1]

Affiliations: Departments of [1]Environmental Health Sciences, [2]Epidemiology, [3]Biostatistics, Mailman School of Public Health, Columbia University, New York NY,10032, USA; [4]Columbia University Arsenic Project in Bangladesh, Dhaka, Bangladesh

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ABSTRACT

Chronic exposure to inorganic arsenic (InAs) through drinking water is a major problem worldwide. InAs undergoes hepatic methylation to form mono- and dimethyl arsenical species (MMA and DMA, respectively), facilitating arsenic elimination. Both reactions are catalyzed by arsenic (+3 oxidation state) methyltransferase (AS3MT) using *S*-adenosylmethionine (SAM) as the methyl donor, yielding the methylated product and *S*-adenosylhomocysteine (SAH), a potent product-inhibitor of AS3MT. SAM biosynthesis depends on folate- and cobalamin-dependent one-carbon metabolism. With the use of samples from 353 participants in the Folate and Oxidative Stress study, our objective was to test the hypotheses that blood SAM and SAH concentrations are associated with arsenic methylation and that these associations differ by folate and cobalamin nutritional status. Blood SAM and SAH were measured by HPLC. Arsenic metabolites in blood and urine were measured by HPLC coupled to dynamic reaction cell inductively coupled plasma MS. In linear regression analyses, SAH was not associated with any of the arsenic metabolites. However, log(SAM) was negatively associated with log(% urinary InAs) (β: -0.11; 95% CI: -0.19, -0.02; $P = 0.01$), and folate and cobalamin nutritional status significantly modified associations between SAM and the percentage of blood MMA (%bMMA) and the percentage of blood DMA (%bDMA) ($P = 0.02$ and $P = 0.01$, respectively). In folate- and cobalamin-deficient individuals, log(SAM) was positively associated with %bMMA (β: 6.96; 95% CI: 1.86, 12.05; $P < 0.01$) and negatively associated with %bDMA (β: -6.19; 95% CI: -12.71, 0.32; $P = 0.06$). These findings suggest that when exposure to InAs is high, and methyl groups are limiting, SAM is used primarily for MMA synthesis rather than for DMA synthesis, contributing additional evidence that nutritional status may explain some of the

interindividual differences in arsenic metabolism and, consequently, susceptibility to arsenic toxicity.

INTRODUCTION

Worldwide, approximately 140 million people are exposed to arsenic at concentrations that exceed the safe drinking water guideline set by the World Health Organization (10 μg/L) [1–3], and >57 million of those exposed live in Bangladesh [4]. Exposure to arsenic is associated with cancers of the skin, lung, bladder, liver, and kidney [5–8] in addition to non-cancer outcomes including peripheral vascular disease [9], atherosclerosis [10], hypertension [11], peripheral neuropathy [12], and decreased intellectual function in children [13]. However, individuals vary in their susceptibility to arsenic-induced health outcomes, and some of this interindividual variation may be explained by differences in arsenic metabolism [14]. In contaminated drinking water, arsenic is present as inorganic arsenic (InAs). Ingested InAs can be methylated to form mono- and dimethyl arsenical species (MMA and DMA, respectively) (**Figure 1**), thereby facilitating arsenic elimination, because DMA has a shorter circulating half-life than does InAs and is rapidly excreted in urine [14]. Arsenic-exposed individuals who have a higher proportion of MMA and a lower proportion of DMA in their urine have an increased risk of developing adverse health outcomes [15]. Therefore, methylation of arsenic to DMA is considered a detoxification pathway [14].

Both steps of arsenic methylation are catalyzed by arsenic (+3 oxidation state) methyltransferase (AS3MT) and require a methyl group from *S*-adenosylmethionine (SAM) [16]. Synthesis of SAM via one-carbon metabolism (OCM) depends on folate in the form of 5-methyltetrahydrofolate (5-mTHF) and cobalamin; the latter acts as a cofactor for methionine

synthase, which catalyzes the transfer of a methyl group from 5-mTHF to homocysteine (Hcys) to generate methionine (See **Supplemental Material**, **Figure S1**). Each methylation step requiring SAM yields the methylated product and *S*-adenosylhomocysteine (SAH) [17]. Importantly, SAH is a potent product-inhibitor of most methyltransferases [18], including AS3MT [19]. Therefore, the concentrations of SAM and SAH and the ratio of SAM to SAH (SAM:SAH) have been used frequently as indicators of methylation capacity [20–22].

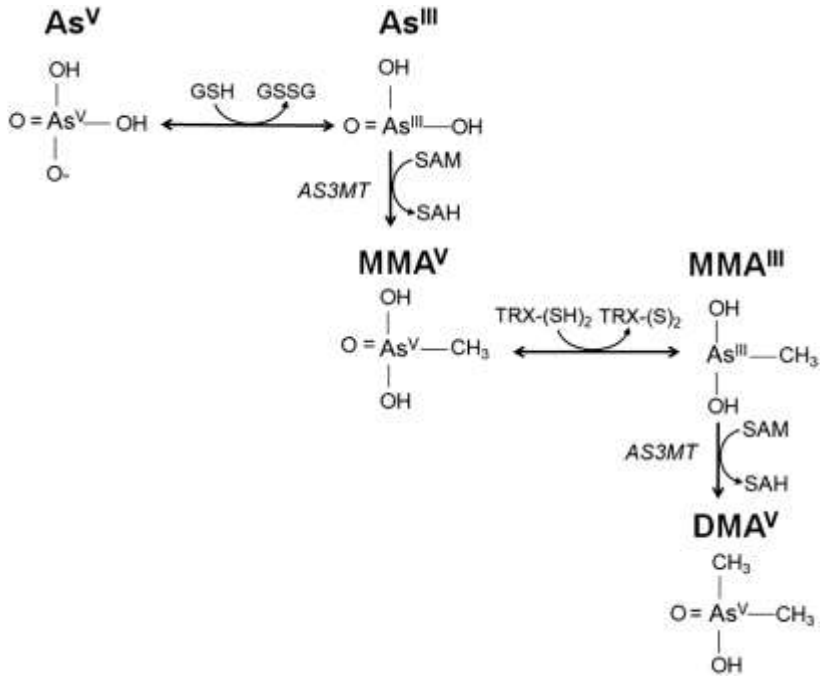

Figure 1. SAM-dependent arsenic metabolism. As^{V} is reduced to As^{III} in a reaction thought to be dependent on GSH or other endogenous reductants. As^{III} then undergoes oxidative methylation, catalyzed by AS3MT and, with SAM as the methyl donor, forms MMA^{V} and SAH. MMA^{V} is reduced to MMA^{III} and can be methylated in a second oxidative methylation step, which is also catalyzed by AS3MT and requires SAM as the methyl donor to produce DMA^{V} and SAH. Abbreviations used: As^{III}, arsenite; As^{V}, arsenate; AS3MT, arsenic (+3 oxidation state) methyltransferase; DMA^{V}, dimethylarsinic acid; GSH, glutathione; GSSG, oxidized glutathione; MMA^{III}, monomethylarsonous acid; MMA^{V}, monomethylarsonic acid; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; TRX, thioredoxin

Previously, our group reported that folic acid (FA) supplementation in folate-deficient adults enhances methylation of InAs to DMA [23]. Because SAM synthesis relies on folate- and cobalamin-dependent OCM (See Supplemental Material, Figure S1), FA supplementation facilitates arsenic methylation by regenerating this methyl donor. However, the relations between blood SAM and SAH and arsenic methylation have not been examined in a human population. Therefore, the objective of this study was to test the hypothesis that blood SAM, like plasma folate, is associated with increased arsenic methylation, whereas blood SAH is associated with decreased arsenic methylation. We hypothesized that SAM would be negatively associated with the percentage of InAs (%InAs) for all participants. However, we predicted that the relations between SAM and the methylated metabolites (%MMA and %DMA) would differ between individuals who were sufficient or deficient for folate and cobalamin, because the relation between SAM and the methylated arsenic metabolites may depend on whether or not SAM is limiting.

STUDY PARTICIPANTS AND METHODS

Study region. Our study site, which is the site of the Health Effects of Arsenic Longitudinal Study (HEALS) cohort [24], is currently a 35-km^2 area within Araihazar, Bangladesh, which is situated ~30 km east of Dhaka.

Participants. The Folate and Oxidative Stress (FOX) study is a cross-sectional study in 378 participants selected from 5 water arsenic (wAs)–exposure categories [<10 μg/L (*n* = 76), 10–100 μg/L (*n* = 104), 101–200 μg/L (*n* = 86), 201–300 μg/L (*n* = 67), and >300 μg/L (*n* = 45) [25]] who were recruited between February and July of 2008. This study had 2 major aims: 1) to study the dose-response relation between wAs exposure and oxidative stress [25, 26] and 2) to study the hypotheses outlined herein. Participants between the ages of 30 and 65 y were eligible.

The following individuals were excluded: 1) women who were pregnant, 2) participants taking nutritional supplements, and 3) participants with known diabetes, cardiovascular or renal disease, or other diseases known to be associated with oxidative stress. Bangladeshi field staff physicians obtained informed consent after reading an approved consent form to study participants. This study was approved by both the Bangladesh Medical Research Council and the Institutional Review Board of Columbia University Medical Center.

General characteristics of study participants. General characteristics of the study participants (**Table 1**) were obtained by questionnaire. Body mass index (BMI) was calculated by using the measured height and weight of each participant. Dietary intakes of folate and cobalamin were determined by food frequency questionnaire [27].

Sample collection and handling. During each participant's visit to our field clinic, a physician collected a venous blood sample. Spot urine samples were collected in 50-mL acid-washed polypropylene tubes and frozen at –20°C. After blood samples underwent initial processing in the field clinic, aliquots of blood and plasma were immediately frozen at -80°C. Samples were then transported on dry ice to Dhaka by car where they were again stored in -80°C (blood and plasma) or -20°C (urine) freezers. In Dhaka, samples were packed on dry ice and flown to Columbia University.

Water arsenic. Field sample collection and laboratory analysis procedures are described elsewhere in detail [28, 29]. Water samples were analyzed by high-resolution inductively coupled plasma MS after 1:10 dilution and addition of Ge to correct fluctuations in instrument sensitivity. The detection limit of the method is typically <0.2 mg/L. Arsenic standards of known concentration were run multiple times in each batch. The intra- and interassay CVs were 6.0% and 3.8%, respectively.

Total blood arsenic. Total blood arsenic (bAs) concentrations were measured by using a Perkin-Elmer Elan DRC II inductively coupled plasma mass spectrometer equipped with an AS 93+ autosampler, as described previously [30]. The intra- and interassay CVs were 3.2% and 5.7%, respectively.

Total urinary arsenic. Total urinary arsenic (uAs) concentrations were measured by graphite furnace atomic absorption spectrometry [31] using the AAnalyst 600 graphite furnace system (PerkinElmer), as previously described [25]. The intra- and interassay CVs were 3.8% and 5.1%, respectively. A method based on the Jaffe reaction was used to measure urinary creatinine (uCr) concentrations [32].

Blood and urine arsenic metabolites. Four arsenic metabolites [arsenite (As^{III}), arsenate (As^{V}), monomethylarsonous acid plus monomethylarsonic acid (MMA^{III+V}), and dimethylarsinous acid plus dimethylarsinic acid (DMA^{III+V})] were measured in blood and urine by coupling HPLC to dynamic reaction cell inductively coupled plasma MS, as described previously [33]. The reduced and oxidized forms of MMA and DMA cannot be separated by HPLC, so each metabolite is measured as one variable (i.e., as MMA^{III+V} or DMA^{III+V}, respectively). All four bAs metabolites could only be measured for individuals with bAs concentrations ≥5 μg/L. Each metabolite (with As^{III} + As^{V} combined as InAs) was calculated as a percentage of the total measured uAs or bAs. The intra-assay CVs for urinary As^{III}, As^{V}, MMA, and DMA were 3.6%, 4.5%, 1.5%, and 0.6%, respectively; those for blood were 0.9%, 11.5%, 3.6%, and 2.6%, respectively. The inter-assay CVs for urinary metabolites were 9.7%, 10.6%, 3.5%, and 2.8%, respectively, whereas those for blood were 3.7%, 23.2%, 2.9%, and 3.5%, respectively.

Plasma folate and cobalamin. A radio-protein-binding assay (SimulTRACS; MP Biomedicals) was used to measure folate and cobalamin, as previously described [25, 33]. The within- and between-day CVs for folate were 9% and 14%, respectively, and for cobalamin these were 5% and 9%, respectively.

Plasma total Hcys. Hcys concentrations were measured in plasma by HPLC with fluorescence detection [34]. The within- and between-day CVs were 2% and 9%, respectively.

SAM and SAH. SAM and SAH were measured as described by Wise et al. [35] in whole blood. Briefly, samples were thawed and mixed on a vortex, and 400 µL of blood was added to 200 µL of 0.1 mol/L sodium acetate, pH 6.0, and 160 µL 40% trichloroacetic acid. After 30 min of incubation on ice, tubes were centrifuged for 10 min at 20,817 x *g*. An aliquot of 200 µL of the supernatant was filtered by using a 0.45-µm Ultra free MC filter (Millipore) and centrifuged for 3 min at 2,655 x *g* for measurement of SAM. For SAH, the remaining supernatant was extracted twice with 100 µL of diethyl ether followed by filtration with another 0.45-µm filter. SAM and SAH were separated by reversed phase HPLC on a 25 x 0.46 cm (5-µm particle size) column (Beckman Instruments) by using a mobile phase consisting of 50 mmol/L NaH_2PO_4 and 10 mmol/L heptane sulfonic acid in 18% methanol, adjusted to pH 4.38 with phosphoric acid at a flow rate of 0.9 mL/min. The running column was preceded by a precolumn filter (ChromTech). By using a 996 Photodiode Array UV absorbance detector (Waters), SAM and SAH were detected at 254 nm and were quantitated by comparing the integrated areas under HPLC peaks with standard curves generated by using purified SAM and SAH (Sigma). The interassay CVs for SAM and SAH were 9.6% and 16.1%, respectively.

Statistical analyses. Of the 378 participants recruited for the FOX study, 353 had complete information for the predictor variables (SAM and SAH), urine outcome variables (%uAs metabolites), and potential confounders. Descriptive statistics were calculated for general characteristics of this study sample, including for arsenic metabolites and nutrition variables; these values are reported as medians (ranges) for continuous variables and as frequencies (%) for categorical variables. The Wilcoxon rank-sum test was used to detect differences in quantitative variables, including SAM and SAH, by dichotomous characteristics, including folate and cobalamin nutritional status (i.e., deficient vs. sufficient). Folate and cobalamin deficiencies were defined by using cutpoints from Christenson et al., as follows: plasma folate <9 nmol/L and plasma cobalamin <151 pmol/L [36]. Spearman correlation coefficients, reported as rho (ρ), were used to assess bivariate relations between quantitative variables including SAM and SAH concentrations, arsenic metabolites, and other continuous measures. To examine the bivariate relations between blood InAs (bInAs) or blood MMA (bMMA) and the ratio of blood DMA (bDMA) to bMMA (bDMA:bMMA), scatterplots and corresponding LOESS curves were plotted in R (R Foundation) using the default smoothing parameter 0.7. Spearman correlations were used to evaluate the statistical significance of the bivariate relations.

Linear regression models were used to evaluate the relation between each of the predictors (SAM, SAH, SAM:SAH) and the outcome variables [% urinary InAs (uInAs), % urinary MMA (uMMA), % urinary DMA (uDMA), %bInAs, %bMMA, %bDMA)]. The estimated regression coefficient for each predictor of interest is reported as β (95% CI) and *P* value. Because bAs metabolites could only be measured for individuals with total bAs concentrations ≥5 μg/L, the sample size for bAs outcomes (%bAs metabolites) was smaller (*n* = 276) than the sample size for uAs outcomes (%uAs metabolites) (*n* = 353). To meet model

assumptions, a natural log transformation (log) was applied to arsenic metabolites with skewed distributions (%uInAs, %uMMA, %bInAs) for approximate normality. To satisfy the linearity assumption for regression models, SAM, SAH, SAM:SAH, age, BMI, folate, and cobalamin were log-transformed, and a square root transformation was applied to wAs. Potential control variables included sex, age, BMI, folate and cobalamin (measured continuously), television ownership (an indicator of socioeconomic status in this population), years of education, cigarette smoking status (ever or never smoker), uCr, estimated glomerular filtration rate, and amount of time (days) that blood samples were stored at -80°C before SAM and SAH analysis by HPLC. All linear regression models were adjusted for age, smoking status, sex, wAs exposure, and blood sample storage time at -80°C. Results were not altered appreciably after further adjusting for television ownership, years of education, BMI, folate, cobalamin, or estimated glomerular filtration rate, so these variables were not included in the final models. The associations between SAM, SAH, SAM:SAH, and arsenic metabolites were also assessed stratified by folate and cobalamin status. The Wald test was used to detect differences between strata in the covariate-adjusted regression coefficient for SAM with respect to methylated bAs metabolites. Because a subset of individuals were deficient for both folate and cobalamin, stratified analyses were also performed in the following four groups: deficient for both nutrients, deficient for folate only, deficient for cobalamin only, sufficient for both nutrients. Due to the small sample size of the group deficient for both folate and cobalamin (n = 32), the final models for the 4 groups were adjusted for a restricted set of variables: sex, wAs, and blood sample storage time at -80°C, because the exclusion of the covariates age and cigarette smoking status from regression models did not appreciably alter the coefficient for SAM. SAS and R were used to conduct all statistical analyses, and a significance level of 0.05 was used.

RESULTS

General characteristics of study participants. Descriptive statistics for general characteristics of the FOX participants are shown in **Table 1**. By design, the participants were between the ages of 30 and 63 y. Approximately 32.9% of the participants were underweight (BMI <18.5 kg/m^2). Median (range) dietary intakes of folate and cobalamin were 241 (86–674) μg/d and 1.32 (0.09–6.25) μg/d, respectively (Table 1); thus, in FOX participants the average dietary intakes of these B vitamins were below the recommended dietary allowances for adults (folate: 400 μg/d; cobalamin: 2.4 μg/d) [37, 38]. Prevalences (%) of folate and cobalamin deficiencies were 30.3% and 34.8%, respectively. Due to the study design, the mean wAs exposure was 140 μg/L, which is 14-fold higher than the World Health Organization standard of 10 μg/L. In blood, on average, the %MMA exceeded the %InAs or %DMA. Conversely, in urine, the mean %DMA was higher than the mean %InAs or %MMA.

SAM and SAH concentrations and relations with other indices of OCM. The relations between SAM, SAH, and other bivariate variables are reported in **Supplemental Material, Table S1**. SAH concentrations were significantly higher in folate-deficient individuals compared with folate-sufficient individuals. SAM concentrations did not differ by folate nutritional status. However, SAM concentrations were significantly higher in cobalamin-sufficient individuals. Correlations between nutrition variables are reported in **Supplemental Material, Table S2**. SAM and folate were not significantly correlated. However, SAM was positively correlated with cobalamin ($r = 0.17$, $P = 0.01$). SAH was negatively correlated with folate ($r = -0.15$, $P < 0.01$) and positively correlated with Hcys ($r = 0.18$, $P < 0.001$).

Table 1. General characteristics of arsenic-exposed, Bangladeshi adults (ages 30-63 y) enrolled in the FOX study

	Value[a]	Median (Range)
Age (y)	43 ± 8	42 (30–63)
Education (y)	3.4 ± 3.6	3.0 (0.0–16.0)
BMI (kg/m^2)	20.4 ± 3.5	19.7 (13.8–35.3)
wAs (μg/L)	140 ± 125	114 (0–700)
uAs (μg/L)	205 ± 229	124 (3–1990)
uCr (mg/dL)	54 ± 43	41 (4–224)
bAs (μg/L)	13.5 ± 9.9	10.8 (1.2–57.0)
bInAs[b] (μg/L)	4.3 ± 2.2	3.8 (1.4–15.8)
bMMA[b] (μg/L)	6.1 ± 3.8	5.1 (0.8–25.1)
bDMA[b] (μg/L)	4.6 ± 2.7	3.8 (1.1–22.7)
bInAs[b] (%)	29.5 ± 4.1	29.4 (19.7–46.7)
bMMA[b] (%)	39.3 ± 5.4	39.7 (20.5–51.5)
bDMA[b] (%)	31.2 ± 5.6	31.3 (18.0–46.5)
uInAs (μg/L)	37 ± 44	22 (0–327)
uMMA (μg/L)	31 ± 39	16 (0–303)
uDMA (μg/L)	140 ± 166	89 (2–1290)
uInAs (%)	17.7 ± 5.6	17.2 (6.7–51.8)
uMMA (%)	14.1 ± 5.0	13.4 (3.6–30.0)
uDMA (%)	68.3 ± 7.9	69.4 (38.3–88.0)
Dietary folate[c] (μg/d)	267 ± 88	241 (86–674)
Dietary cobalamin[c] (μg/d)	1.50 ± 0.77	1.32 (0.09–6.25)
Plasma folate (nmol/L)	12.8 ± 7.3	11.1 (2.4–60.6)
Plasma cobalamin (pmol/L)	203 ± 113	176 (44–1180)
Plasma Hcys (μmol/L)	11 ± 13	9 (3–165)
Blood SAM (μmol/L)	1.30 ± 0.53	1.18 (0.44–3.69)
Blood SAH (μmol/L)	0.31 ± 0.18	0.27 (0.07–1.37)
Male (%)	50.7	—

Ever smoker (%)	38.2	—
Ever used betel nut (%)	43.1	—
Own television (%)	58.4	—
Underweight[d] (%)	32.9	—
Folate deficient[e] (%)	30.3	—
Cobalamin deficient[f] (%)	34.8	—

Abbreviations used: bAs, blood arsenic; bInAs, blood inorganic arsenical species; bDMA, blood dimethyl arsenical species; BMI, body mass index; bMMA, blood monomethyl arsenical species; Hcys, homocysteine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; uAs, urinary arsenic; uCr, urinary creatinine; uDMA, urinary dimethyl arsenical species; uInAs, urinary inorganic arsenical species; uMMA, urinary monomethyl arsenical species; wAs, water arsenic

[a]Values are means ± SD or percentages; $n = 353$.
[b]$n = 276$
[c]$n = 347$
[d]BMI <18.5 kg/m^2
[e]Plasma folate <9 nmol/L
[f]Plasma cobalamin <151 pmol/L

Correlations between nutrition variables and arsenic metabolites. Correlations between nutrition variables and arsenic metabolites are reported in **Table 2**. Folate was negatively correlated with %InAs and %MMA and positively correlated with %DMA in blood and urine. In contrast, Hcys was positively correlated with %MMA and negatively correlated with %DMA in blood and urine. Cobalamin was positively correlated with %MMA in urine but not in blood. SAM was positively correlated with %MMA in blood and urine and negatively correlated with %uInAs. SAH was not correlated with any of the arsenic metabolites.

Table 2. Correlations[a] between nutrition variables and arsenic metabolites in arsenic-exposed, Bangladeshi adults (ages 30-63 y) enrolled in the FOX study

	Blood Metabolites (*n* = 276)			**Urinary Metabolites (*n* = 353)**		
	%InAs	%MMA	%DMA	%InAs	%MMA	%DMA
Folate[b] (nmol/L)	−0.14*	−0.22$^{\#}$	0.29$^{\#}$	−0.15**	−0.21$^{\#}$	0.24$^{\#}$
Cobalamin[c] (pmol/L)	−0.06	−0.04	0.07	−0.10	0.12*	−0.03
Hcys (µmol/L)	0.05	0.20**	−0.22$^{\#}$	−0.03	0.22$^{\#}$	−0.13*
SAM (µmol/L)	−0.03	0.13*	−0.08	−0.11*	0.12*	−0.01
SAM[d] (µmol/L)	−0.03	0.13*	−0.08	−0.11	0.12*	−0.01
SAH (µmol/L)	−0.04	0.01	0.02	−0.06	0.02	0.04
SAH[d] (µmol/L)	0.05	−0.07	0.03	−0.04	0.02	0.01
BMI (kg/m^2)	−0.09	−0.06	0.12	−0.06	−0.18$^{\#}$	0.15**

Abbreviations used: BMI, body mass index; %DMA, proportion of dimethyl arsenical species; FOX, Folate and Oxidative Stress study; Hcys, homocysteine; %InAs, proportion of inorganic arsenical species; %MMA, proportion of monomethyl arsenical species; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine

*$P < 0.05$, **$P < 0.01$, $^{\dagger}P < 0.001$, $^{\ddagger}P < 0.0001$.

[a]Spearman correlation coefficients.
[b]Folate measured continuously
[c]Cobalamin measured continuously
[d]Partial Spearman correlation, adjusted for blood sample storage time at -80°C.

Relation between blood arsenic metabolites. bDMA:bMMA decreased with increasing concentrations of bInAs or bMMA (**Figure 2**). The Spearman correlations between bInAs or bMMA and bDMA:bMMA were negative and significant ($P < 0.05$).

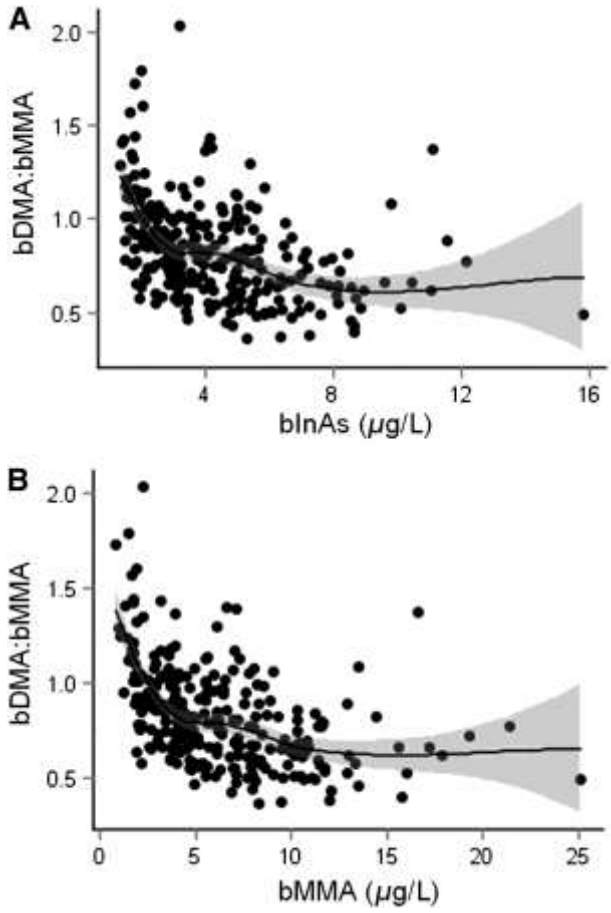

Figure 2. Scatterplots and LOESS curves showing relations between bDMA:bMMA and bInAs (**A**) or bMMA (**B**) in arsenic-exposed, Bangladeshi adults (ages 30-63 y) enrolled in the FOX study ($n = 353$). The black line is the smoothed regression line, with bDMA:bMMA as the outcome and bInAs or bMMA concentration (μg/L) as the predictor. The gray shading is the corresponding 95% confidence band. Abbreviations used: bDMA:bMMA, ratio of dimethyl to monomethyl arsenical species in blood; bInAs, blood inorganic arsenical species; bMMA, blood monomethyl arsenical species; FOX, Folate and Oxidative Stress study

***SAM, SAH, and SAM:SAH as predictors of arsenic methylation*.** In regression analyses, after adjusting for age, sex, wAs, cigarette smoking status, and blood sample storage time at -80°C, there was a significant negative association between log(SAM) and log(%uInAs) (β: -0.11; 95% CI: -0.19, -0.02; $P = 0.01$) (**Table 3**). No other significant associations were observed between SAM and the arsenic metabolites (Table 3). SAH and SAM:SAH were not significantly associated with any of the arsenic metabolites (Table 3). The results from the urinary metabolite analyses were very similar after restricting the sample size to participants with measured blood arsenic metabolites. The results were not appreciably altered when SAM and SAH were included simultaneously in the regression models, nor were the results altered after adjusting for folate or cobalamin as continuous predictors in the model. In analyses stratified by folate status, log(SAM) was positively and significantly associated with %bMMA in those who were folate deficient (β: 3.49; 95% CI: 0.40, 6.59; $P = 0.03$) but not in those who were folate sufficient (β: -0.43; 95% CI: -2.47, 1.61; $P = 0.68$) (**Table 4**). Stratifying by cobalamin status revealed similar findings; although not statistically significant, log(SAM) was positively associated with %bMMA in those who were deficient for cobalamin (β: 2.58; 95% CI: -0.44, 5.60; $P = 0.09$) but not in those who were sufficient for cobalamin (β: 0.15; 95% CI: -1.93, 2.24; $P = 0.90$). The Wald test for the difference in the association between SAM and %bMMA across folate strata was significant ($P = 0.04$). SAM was also negatively associated with %bDMA in the cobalamin-deficient group ($P = 0.01$); this was not observed in the folate-deficient group. The association between SAM and %bDMA differed significantly between cobalamin strata ($P < 0.01$). Although the patterns of association were similar for uAs metabolites, the positive associations between log(SAM) and log(%MMA) in folate-deficient participants (β: 0.17; 95% CI: -0.01, 0.35; $P = 0.07$) and cobalamin-deficient participants (β: 0.15; 95% CI: -0.03, 0.32; $P = 0.10$) did

not achieve statistical significance (**Supplemental Material, Tables S3** and **S4**). When individuals were stratified into four groups on the basis of joint folate and cobalamin status, there was a strong, positive association between log(SAM) and %bMMA in individuals who were deficient for both folate and cobalamin (β: 6.96; 95% CI: 1.86, 12.05; $P < 0.01$) (**Table 5**). The Wald test for overall differences in the association between SAM and %bMMA across the four nutrition groups was significant ($P = 0.02$), mainly due to the difference between those who were deficient for both folate and cobalamin and those who were sufficient for the two nutrients ($P < 0.01$). In individuals deficient for cobalamin and in individuals deficient for both folate and cobalamin, there was a negative association between SAM and %bDMA, although these associations were not significant ($0.05 < P < 0.10$). However, the Wald test for overall differences in the association between SAM and %bDMA across the four nutrition groups was significant ($P = 0.01$) and was driven by the differences between those who were sufficient for both nutrients and those who were deficient for both nutrients ($P < 0.01$) or deficient for cobalamin only ($P = 0.02$).

DISCUSSION

In vitro [16, 39] and animal [40] studies have established that SAM is necessary for the methylation of InAs to MMA and for the methylation of MMA to DMA and that SAH inhibits both of these methylation steps [19]. However, the relations between SAM and the percentage of arsenic metabolites in human populations may be particularly complex for 2 reasons: 1) there is competition between InAs and MMA for methylation, because both methylation steps are catalyzed by AS3MT and require a methyl group from SAM, and 2) these relations may depend on nutritional status, because SAM is synthesized via folate- and cobalamin-dependent OCM. In

Table 3. Associations between SAM, SAH, SAM:SAH, and arsenic metabolites in arsenic-exposed Bangladeshi adults (ages 30-63 y) enrolled in the FOX study[a]

	Log(%InAs)		**%MMA**[b]		**%DMA**	
Blood (*n* = 280)	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*
log(SAM)	−0.01 (−0.05, 0.04)	0.75	0.77 (−0.90, 2.45)	0.36	−0.35 (−2.19, 1.49)	0.71
log(SAH)	0.02 (−0.02, 0.05)	0.37	−0.70 (−2.03, 0.62)	0.30	0.17 (−1.28, 1.63)	0.82
log(SAM:SAH)	−0.02 (−0.05, 0.02)	0.33	0.86 (−0.26, 1.99)	0.13	−0.28 (−1.52, 0.96)	0.65
Urine (*n* = 359)						
log(SAM)	−0.11 (−0.19, −0.02)	0.01	0.06 (−0.04, 0.15)	0.23	0.87 (−1.28, 3.01)	0.43
log(SAH)	−0.03 (−0.10, 0.04)	0.35	0.01 (−0.06, 0.09)	0.77	0.59 (−1.12, 2.29)	0.50
log(SAM:SAH)	−0.03 (−0.09, 0.03)	0.35	0.02 (−0.05, 0.08)	0.56	−0.03 (−1.53, 1.47)	0.97

Abbreviations used: %DMA, proportion of dimethyl arsenical species; FOX, Folate and Oxidative Stress Study; %InAs, proportion of inorganic arsenical species; %MMA, proportion of monomethyl arsenical species; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; wAs, water arsenic

[a]Reported β values (95% CIs) and *P* values were determined from linear regression analyses, adjusted for log(age), sex, cigarette smoking status, square root (wAs), and SAM/SAH blood sample storage time at -80°C
[b]%MMA in urine was log-transformed

Table 4. Associations[a] between SAM and methylated blood arsenic metabolites in arsenic-exposed Bangladeshi adults (ages 30-63 y) enrolled in the FOX study, stratified by folate or cobalamin nutritional status

	Log(SAM) and %bMMA		**Log(SAM) and %bDMA**	
	β (95%CI)	*P*	β (95%CI)	*P*
Folate status[b]		0.04[c]		0.44[c]
Folate sufficient (*n* = 194)	−0.43 (−2.47, 1.61)	0.68	0.14 (−1.98, 2.26)	0.89
Folate deficient (*n* = 86)	3.49 (0.40, 6.59)	0.03	−1.51 (−5.22, 2.19)	0.42
Cobalamin status[d]		0.19[c]		<0.01[c]
Cobalamin sufficient (*n* = 179)	0.15 (−1.93, 2.24)	0.88	0.99 (−1.28, 3.25)	0.39
Cobalamin deficient (*n* = 101)	2.58 (−0.44, 5.60)	0.09	−4.33 (−7.54, −1.12)	0.01

Abbreviations used: FOX, Folate and Oxidative Stress Study; SAM, *S*-adenosylmethionine; wAs, water arsenic

[a]Reported β values (95% CIs) and *P* values were determined from linear regression analyses, adjusted for log(age), sex, cigarette smoking status, square root (wAs), and SAM blood sample storage time at -80°C
[b]Folate deficient = plasma folate <9 nmol/L; folate sufficient = plasma folate ≥9 mol/L
[c]Wald test for difference in covariate-adjusted β between strata.
[d]Cobalamin deficient = plasma cobalamin <151 pmol/L; cobalamin sufficient = plasma cobalamin ≥151 pmol/L

Table 5. Associations[a] between SAM and methylated blood arsenic metabolites in arsenic-exposed Bangladeshi adults (ages 30-63 y) enrolled in the FOX study, stratified by joint folate and cobalamin nutritional status.

	Log(SAM) and %bMMA		**Log(SAM) and %bDMA**	
Group[b]	β (95% CI)	*P*	β (95% CI)	*P*
Sufficient for both (*n* = 126)	−1.15 (−3.55, 1.25)	0.34	1.70 (−0.91, 4.31)	0.20
Deficient for folate (*n* = 52)	2.26 (−1.38, 5.90)	0.22	0.45 (−3.57, 4.46)	0.82
Deficient for cobalamin (*n* = 66)	0.61 (−3.29, 4.52)	0.76	−3.48 (−7.43, 0.46)	0.08
Deficient for both (*n* = 32)	6.96 (1.86, 12.05)	<0.01	−6.19 (−12.71, 0.32)	0.06
P for all groups		0.02[c]		0.01[c]

Abbreviations used: FOX, Folate and Oxidative Stress study; SAM, *S*-adenosylmethionine; wAs, water arsenic

[a]Reported β values (95% CIs) and *P* values were determined from linear regression analyses, adjusted for sex, square root(wAs), and SAM blood sample storage time at -80°C.
[b]Deficient for folate = plasma folate <9 nmol/L; deficient for cobalamin= plasma cobalamin <151 pmol/L
[c]Wald test for overall difference between the 4 groups

a cross-sectional study in arsenic-exposed adults in Bangladesh, our group previously observed that folate was negatively correlated with %InAs and %MMA and positively correlated with %DMA in urine, suggesting that folate facilitates the methylation of InAs to DMA [41]; we also observed this in the current study. However, in our previous study, we did not analyze blood SAM and SAH concentrations.

With the use of purified recombinant human AS3MT, Song et al. [42] demonstrated that when SAM concentrations are <0.5 mmol/L, the rate of MMA synthesis exceeds the rate of DMA synthesis. Although there are few estimates of human liver SAM concentrations in healthy individuals, reported SAM concentrations in rat liver vary from 60 to 160 μmol/L [43–45]. Therefore, according to the findings of Song et al. [42], the rate of MMA production should exceed the rate of DMA production at physiologically relevant concentrations of SAM. Consistent with this, we observed that the mean %MMA exceeded the mean %DMA in blood. Song et al. [42] also observed that with increasing InAs concentrations, the %MMA increases, the %DMA decreases, and the ratio of DMA to MMA decreases. Similarly, Styblo et al. [46] demonstrated that human hepatocytes exposed to increasing concentrations of InAs produce more MMA and less DMA. These studies indicate that the second step of arsenic methylation is inhibited by InAs. Evidence from mathematical modeling also suggests that MMA can inhibit its own methylation, likely due to substrate inhibition (Michael Reed and Fred Nijhout, Duke University, personal communication). Thus, methylation of InAs to MMA may predominate over the methylation of MMA to DMA in individuals who are continuously exposed to high concentrations of InAs, such as the current study participants, because 1) high amounts of InAs compete with MMA for methylation and 2) the second methylation step is inhibited by MMA. Our finding of a decrease in bDMA:bMMA with increasing concentrations of bInAs and bMMA

is consistent with this. Furthermore, our observation that SAM is positively associated with %bMMA but only in folate- and cobalamin-deficient participants suggests that limiting SAM concentrations further reduces the ability to methylate MMA to DMA.

Although we had anticipated a strong positive association between SAM and %bDMA in folate- and cobalamin-sufficient participants, many of the FOX participants were drinking from wells with very high wAs concentrations; thus, the null association between SAM and %bDMA in sufficient participants may reflect the strong inhibition of DMA synthesis by InAs and MMA. Alternatively, it may reflect saturation of the AS3MT at higher concentrations of SAM. Although Song et al. [42] observed that within the range of physiologically relevant concentrations of SAM, DMA production increases with increasing SAM concentrations, glutathione was the only reductant used in their assays; other groups have demonstrated that arsenic methylation is more efficient in the presence of other reductants, such as thioredoxin [47]. Thus, saturation of AS3MT may occur at lower physiologically relevant concentrations of SAM.

Because folate and cobalamin are involved in SAM synthesis, a simplistic prediction was that both folate and cobalamin would be positively correlated with blood SAM concentrations. However, SAM was not correlated with plasma folate in the FOX participants. Increasing SAM concentrations leads to inhibition of methylenetetrahydrofolate reductase through long-range allosteric interactions, such that production of 5-mTHF decreases when SAM concentrations increase. This negative feedback loop may explain why there is no observable correlation between plasma 5-mTHF and SAM in our study. Loehrer et al. [48] similarly observed no correlation between SAM and 5-mTHF in their case-control study examining the relation between folate and coronary artery disease. As expected, in the FOX participants, plasma folate

was negatively correlated with both SAH and plasma Hcys concentrations and was positively correlated with %DMA in blood and urine. Additionally, cobalamin was positively correlated with SAM, and cobalamin-sufficient individuals had significantly higher SAM concentrations than did cobalamin-deficient individuals. This finding is reasonable given that, unlike folate, cobalamin concentrations are not regulated by SAM.

We did not find SAH to be significantly associated with any of the arsenic metabolites, which was surprising given that SAH is a potent inhibitor of most SAM-dependent methylation reactions *in vitro* [18]. However, it is important to note that we measured SAM and SAH in blood, yet arsenic methylation primarily occurs in the liver. Although blood SAM and SAH concentrations are considered indicators of methylation capacity and have been used as biomarkers in several other studies [49–51], we are unaware of any studies that have directly compared liver and whole-blood SAM and SAH concentrations. Although Hcys is readily exported from cells [52], the transport of intact SAH across the plasma membrane is not well characterized [53]. James et al. [53] proposed that Hcys may, in fact, serve as an exportable form of SAH, because it is more readily transported out of the cell [54] and may therefore be a better indicator of liver SAH concentrations than is blood SAH. As in previous studies, we found plasma Hcys to be positively correlated with %MMA in blood and urine and negatively correlated with %bDMA. If plasma Hcys is indeed a better indicator of hepatic SAH concentrations than is blood SAH itself, these findings are consistent with inhibition of the second methylation step of MMA to DMA by SAH in the liver.

The findings of this study have three major implications. First, the null associations between SAH and the arsenic metabolites highlight the need for additional research examining the utility of blood SAM and SAH (or alternatively plasma Hcys) as biomarkers of liver SAM

and SAH. Second, the inverse relation between either InAs or MMA and the ratio of DMA to MMA indicates that arsenic metabolism may not be as efficient in populations that are continuously exposed to high concentrations of InAs. Third, the observed positive association between SAM and %bMMA in folate- and cobalamin-deficient individuals suggests that these individuals may be particularly susceptible to arsenic-induced toxicity, because a higher %MMA in urine has been associated with multiple adverse health outcomes [15]. Previously, we observed that folate deficiency and hyperhomocysteinemia are risk factors for arsenic-induced skin lesions [55]. The findings of this study contribute additional evidence that folate and cobalamin deficiencies, and hyperhomocysteinemia, may help to explain a portion of the interindividual variation in arsenic methylation capacity and in susceptibility to arsenic toxicity. Although eliminating arsenic exposure should remain the primary target for reducing its toxicity, this work and previous studies collectively indicate a significant need for public health interventions directed toward alleviating these micronutrient deficiencies, particularly in arsenic-exposed populations.

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CHAPTER THREE SUPPLEMENTAL MATERIAL

Table S1. Bivariate analyses for SAM and SAH in arsenic-exposed, Bangladeshi adults (Ages 30-63 y) enrolled in the FOX study

Covariate (*n*)	SAM[a] (µmol/L)	*P*[b]	SAH[a] (µmol/L)	*P*[b]
Males (179)	1.25 (0.44-3.69)		0.30 (0.10-1.37)	
Females (174)	1.13 (0.52-3.38)		0.24 (0.07-1.16)	
		<0.01		<0.01
Owns TV (206)	1.23 (0.57-3.38)		0.25 (0.07-1.16)	
No TV (147)	1.11 (0.44-3.69)		0.29 (0.10-1.37)	
		<0.01		0.22
Ever Smoker (135)	1.28 (0.57-3.69)		0.29 (0.10-1.37)	
Never Smoker (218)	1.12 (0.44-3.38)		0.25 (0.07-1.16)	
		<0.01		0.29
Ever Betel[c] (152)	1.17 (0.57-3.20)		0.28 (0.07-0.95)	
Never Betel[c] (201)	1.19 (0.44-3.69)		0.25 (0.07-1.37)	
		0.55		0.51
Folate Sufficient[d] (246)	1.15 (0.57-3.69)		0.25 (0.07-1.37)	
Folate Deficient[d] (107)	1.25 (0.44-3.03)		0.30 (0.10-0.82)	
		0.21		0.01
Cobalamin Sufficient[e] (230)	1.23 (0.44-3.69)		0.28 (0.07-1.16)	
Cobalamin Deficient[e] (123)	1.06 (0.57-3.38)		0.26 (0.07-1.37)	
		<0.01		0.25

Abbreviations used: FOX, Folate and Oxidative Stress study; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine

[a]Values are median (range)
[b]Wilcoxon rank sum test for difference
[c]Ever Betel = Ever chewed betel nut, Never Betel = Never chewed betel nut
[d]Folate Sufficient = Plasma folate ≥9 nmol/L, Folate Deficient = Plasma folate <9 nmol/L
[e]Cobalamin Sufficient = Plasma cobalamin ≥151 pmol/L, Cobalamin Deficient = Plasma cobalamin <151 pmol/L

Table S2. Correlations[a] between continuous nutrition variables in arsenic-exposed Bangladeshi adults (ages 30-63 y) enrolled in the FOX study (n = 353)

	SAM (µmol/L)	SAH (µmol/L)	Folate (nmol/L)	Hcys (µmol/L)	Cobalamin (pmol/L)
SAH (µmol/L)	0.14**				
Folate (nmol/L)	-0.06	-0.15**			
Hcys (µmol/L)	0.14**	0.18***	-0.45#		
Cobalamin (pmol/L)	0.17**	0.08	0.10	-0.09	
uCr (mg/dL)	0.03	-0.26#	0.06	-0.04	-0.11*
BMI (kg/m^2)	0.09	0.04	0.18#	-0.01	0.03

Abbreviations used: BMI, body mass index; FOX, Folate and Oxidative Stress study; Hcys, homocysteine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; uCr, urinary creatinine

[a]Spearman correlations
*P < 0.05, **P < 0.01, ***P < 0.001, #P < 0.0001

Table S3. Associations[a] between SAM, SAH, SAM:SAH, and arsenic metabolites in arsenic-exposed Bangladeshi adults (ages 30-63 y) enrolled in the FOX study, stratified by folate nutritional status

	Blood[b]			Urine[c]		
	%InAs[d]	**%MMA**	**%DMA**	**%InAs[d]**	**%MMA[d]**	**%DMA**
Folate Sufficient[e]						
SAM[d] β (95%CI)	0.01 (-0.04, 0.07)	-0.43 (-2.47, 1.61)	0.14 (-1.98, 2.26)	-0.11 (-0.21, -0.02)	0.01 (-0.10, 0.12)	1.52 (-0.90, 3.94)
P	0.65	0.68	0.89	0.02	0.88	0.22
SAH[d] β (95%CI)	0.02 (-0.02, 0.06)	-0.29 (-1.81, 1.24)	-0.38 (-1.97, 1.21)	-0.04 (-0.12, 0.04)	0.00 (-0.09, 0.08)	0.67 (-1.20, 2.53)
P	0.32	0.71	0.64	0.32	0.93	0.48
SAM:SAH[d] β (95%CI)	-0.01 (-0.05, 0.03)	0.04 (-1.37, 1.44)	0.39 (-1.07, 1.85)	-0.02 (-0.10, 0.05)	0.01 (-0.07, 0.09)	0.20 (-1.54, 1.94)
P	0.55	0.96	0.60	0.49	0.85	0.82
Folate Deficient[f]						
SAM[d] β (95%CI)	-0.05 (-0.14, 0.04)	3.49 (0.40, 6.59)	-1.51 (-5.22, 2.19)	-0.07 (-0.23, 0.09)	0.17 (-0.01, 0.35)	-0.96 (-5.36, 3.43)
P	0.26	0.03	0.42	0.39	0.07	0.66
SAH[d] β (95%CI)	0.02 (-0.06, 0.11)	-1.58 (-4.36, 1.21)	0.92 (-2.35, 4.18)	-0.01 (-0.15, 0.13)	0.08 (-0.08, 0.24)	-0.19 (-3.98, 3.59)
P	0.55	0.26	0.58	0.90	0.30	0.92
SAM:SAH[d] β (95%CI)	-0.03 (-0.09, 0.02)	2.20 (0.25, 4.16)	-1.07 (-3.41, 1.26)	-0.03 (-0.13, 0.08)	0.03 (-0.10, 0.15)	-0.31 (-3.24, 2.62)
P	0.26	0.03	0.36	0.63	0.68	0.83

Abbreviations used: %DMA, proportion of dimethyl arsenical species; FOX, Folate and Oxidative Stress study; %InAs, proportion of inorganic arsenical species; %MMA, proportion of monomethyl arsenical species; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SAM:SAH, ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine; wAs, water arsenic

[a]Reported β (95%CI) and *P* are from linear regression analyses, adjusted for log(age), sex, cigarette smoking status, square root(wAs), and SAM/SAH sample storage time at -80°C, and stratified by folate nutritional status
[b]*n* for blood metabolites in folate sufficient = 192, in folate deficient *n* = 84
[c]*n* for urine metabolites in folate sufficient = 246, in folate deficient *n* =107
[d]Natural log-transformed
[e]Folate Sufficient defined as plasma folate ≥9 nmol/L
[f]Folate Deficient defined as plasma folate <9 nmol/L

Table S4. Associations[a] between SAM, SAH, SAM:SAH, and arsenic metabolites in arsenic-exposed Bangladeshi adults (ages 30-63 y) enrolled in the FOX study, stratified by cobalamin nutritional status

	Blood[b]			Urine[c]		
	%InAs[d]	**%MMA**	**%DMA**	**%InAs[d]**	**%MMA[d]**	**%DMA**
Cobalamin Sufficient[e]						
SAM[d] β (95%CI)	-0.03 (-0.09, 0.03)	0.15 (-1.93, 2.24)	0.99 (-1.28, 3.25)	-0.13 (-0.23, -0.02)	0.01 (-0.11, 0.12)	1.92 (-0.76, 4.59)
P	0.29	0.88	0.39	0.02	0.92	0.16
SAH[d] β (95%CI)	0.03 (-0.01, 0.08)	-1.38 (-3.08, 0.33)	0.29 (-1.57, 2.16)	-0.01 (-0.10, 0.08)	0.00 (-0.09, 0.09)	0.31 (-1.91, 2.53)
P	0.15	0.11	0.76	0.77	0.99	0.78
SAM:SAH[d] β (95%CI)	-0.04 (-0.07, 0.00)	0.99 (-0.41, 2.38)	0.25 (-1.27, 1.77)	-0.05 (-0.13, 0.02)	0.00 (-0.08, 0.08)	0.70 (-1.16, 2.56)
P	0.06	0.16	0.75	0.18	0.95	0.46
Cobalamin Deficient[f]						
SAM[d] β (95%CI)	0.06 (-0.02, 0.15)	2.58 (-0.44, 5.60)	-4.33 (-7.54, -1.12)	-0.03 (-0.17, 0.11)	0.15 (-0.03, 0.32)	-1.71 (-5.55, 2.12)
P	0.14	0.09	0.01	0.66	0.10	0.38
SAH[d] β (95%CI)	0.01 (-0.05, 0.07)	0.01 (-2.22, 2.24)	-0.20 (-2.62, 2.22)	-0.04 (-0.14, 0.06)	0.01 (-0.12, 0.14)	0.76 (-2.05, 3.56)
P	0.79	0.99	0.87	0.45	0.84	0.59
SAM:SAH[d] β (95%CI)	0.02 (-0.04, 0.08)	1.09 (-0.90, 3.09)	-1.69 (-3.85, 0.46)	0.02 (-0.08, 0.12)	0.06 (-0.06, 0.18)	-1.48 (-4.11, 1.15)
P	0.47	0.28	0.12	0.69	0.35	0.27

Abbreviations used: %DMA, proportion of dimethyl arsenical species; FOX, Folate and Oxidative Stress study; %InAs, proportion of inorganic arsenical species; %MMA, proportion of monomethyl arsenical species; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SAM:SAH, ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine; wAs, water arsenic

[a]Reported β (95%CI) and *P* are from linear regression analyses, adjusted for log(age), sex, cigarette smoking status, square root(wAs), and SAM/SAH sample storage time at -80°C, and stratified by cobalamin nutritional status
[b]*n* for blood metabolites in cobalamin sufficient = 178, in cobalamin deficient *n* = 98
[c]*n* for urine metabolites in cobalamin sufficient = 230, in cobalamin deficient *n* = 123
[d]Natural log-transformed
[e]Cobalamin Sufficient defined as plasma cobalamin ≥151 pmol/L
[f]Cobalamin Deficient defined as plasma cobalamin <151 pmol/L

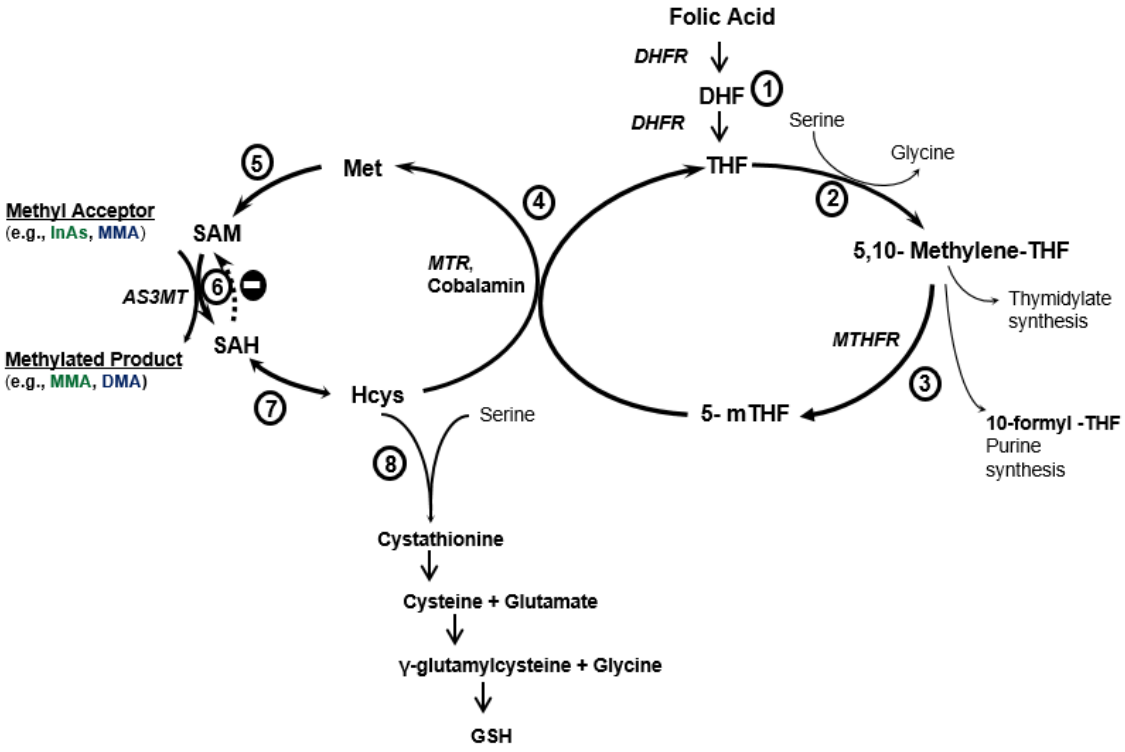

Figure S1. One-carbon metabolism. **(1)** Dihydrofolate reductase (DHFR) reduces dietary folates to dihydrofolate (DHF) and tetrahydrofolate (THF). **(2)** Serine hydroxymethyltransferase transfers the β-carbon of serine to THF, forming 5,10-methenyl-THF and glycine. **(3)** At a major branch point between transmethylation reactions and nucleotide biosynthesis, 5,10-methenyl-THF can be reduced to 5,10-methylene-THF and further reduced to 5-methyl-THF (5-mTHF) by 5,10-methylene-THF reductase (MTHFR). **(4)** Methionine synthase (MTR), a cobalamin-dependent enzyme, catalyzes the transfer of a methyl group from 5-mTHF to homocysteine (Hcys), generating methionine and regenerating THF. **(5)** Methionine adenosyl-transferase activates methionine to form *S*-adenosylmethionine (SAM). **(6)** SAM serves as a universal methyl donor for numerous acceptors, including inorganic (InAs) and monomethylarsonous acid (MMA), yielding MMA and dimethylarsinic acid (DMA), respectively. Matching colors indicate methyl acceptors and their corresponding methylated products. Both arsenic methylation steps are catalyzed by arsenic (+3) methyltransferase (AS3MT). Upon donating a methyl group, SAM is converted to *S*-adenosylhomocysteine (SAH), a potent inhibitor of most SAM-dependent methylation reactions. **(7)** SAH is hydrolyzed to generate Hcys, which is used to regenerate methionine or is **(8)** directed to the transsulfuration pathway through which it is ultimately catabolized for the synthesis of glutathione (GSH).

CHAPTER FOUR

Enzymatic cleavage of histone H3: a new consideration when measuring histone modifications in human samples

Caitlin G. Howe[1] and Mary V. Gamble[1]

Affiliations: [1]Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York NY, 10032, USA

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ABSTRACT:

Posttranslational histone modifications (PTHMs) are increasingly being used as biomarkers of cancer prognosis and survival. However, we identified a specific cleavage product of histone H3 in human peripheral blood mononuclear cells, which interferes with measures of certain H3 modifications. Therefore, the potential for enzymatic cleavage of histones should be considered when measuring PTHMs in human samples. Furthermore, enzymatic cleavage of human H3 is itself a fascinating area of research and two important questions remain to be answered: 1) Does cleavage of human H3 occur *in vivo*, as it does in other organisms? and 2) Does it serve a biologically important function?

ENZYMATIC CLEAVAGE OF HISTONES:

Posttranslational histone modifications (PTHMs) are increasingly being used as biomarkers of cancer prognosis [1]. However, histones are very sensitive to enzymatic degradation by proteases [2], and there is evidence from many organisms that histones are enzymatically cleaved *in vivo*; this topic is receiving increasing attention and has been reviewed recently by several groups [3-5]. Enzymatic cleavage of H3 has been observed in tetrahymena [6], yeast [7-8], chicken [9], quail [10], and mouse [11,12]. Furthermore, certain viruses can cleave host cell H3 [13,14], and antimicrobial peptides derived from the N-terminal regions of various histones (e.g., H2A, H2B, H1) have been identified in several organisms, including fish [15-20], molluscs [21,22], frogs [23], and even from the gastrointestinal tract [24] and wound fluids [25] of humans.

Until recently, there were few reports of histone cleavage in human cells. However, last year, Vossaert et al. reported histone H3 clipping in human embryonic stem cell (ESC)

lines [26], and our group recently identified a cleavage product of H3 in human peripheral blood mononuclear cells (PBMCs) (**Figure 1**).

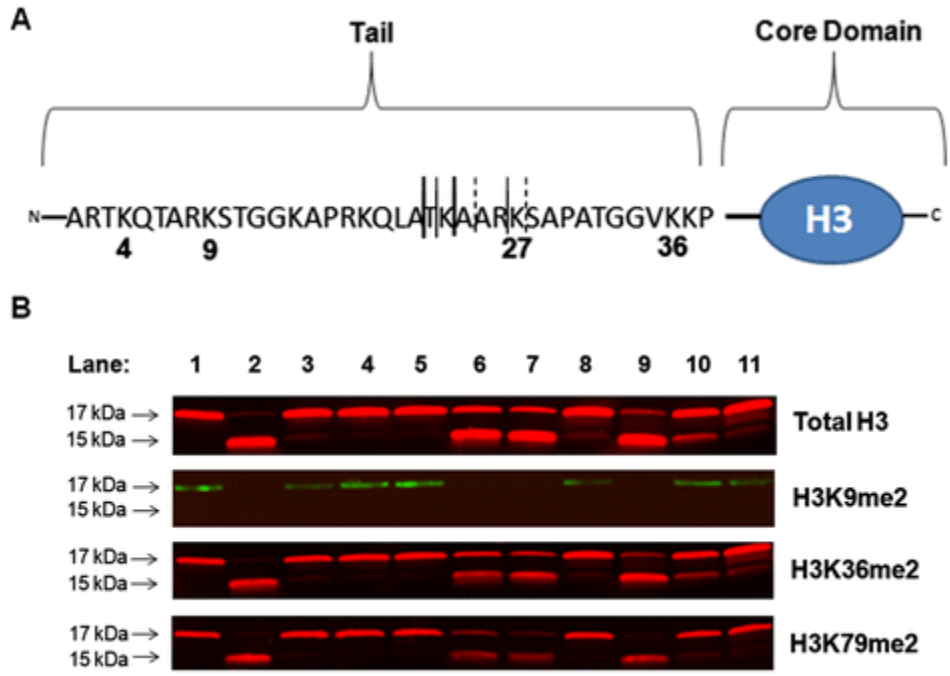

Figure 1. Enzymatic cleavage of H3 interferes with the measurement of certain PTHMs. **(A)** Known enzymatic cleavage sites in H3 for mouse ESCs [11]. Bold solid lines indicate sites that are frequently cleaved, thin solid lines indicate sites that are less frequently cleaved, and dotted lines indicate sites that are rarely cleaved [11]. **(B)** Western blot (Odyssey® CLx Infrared Imaging System, Li-Cor) was used to measure total H3 protein levels (Sigma, H0164, 1:4,000) in 11 representative histone samples that had been isolated, using an acid-extraction method [27], from PBMCs collected from arsenic-exposed Bangladeshi adults enrolled in the FACT study, a randomized controlled trial of folic acid and creatine supplementation; sample collection and processing for this study has been described previously [28]. The expected size of H3 is ~17 kDa. A distinct cleavage product of H3 is observed at ~15 kDa, and an additional H3 cleavage product between 15 and 17 kDa is also present in several of the samples (top panel). In the same 11 samples, three PTHMs that are located in different regions of H3 were assessed by Western blot: H3K9me2 (Abcam, ab1220, 1:1,000, mouse) (second panel), H3K36me2 (Abcam, ab9049, 1:1,000, rabbit) (third panel), and H3K79me2 (Abcam, ab3594, 1:400, rabbit) (fourth panel). Abbreviations used: ESC, embryonic stem cell; FACT, Folic Acid and Creatine Trial; H3K9me2, di-methylation at lysine 9 of histone H3; H3K36me2, di-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; PBMC, peripheral blood mononuclear cell; PTHM, posttranslational histone modification

We observe this H3 cleavage product in spite of the use of protease inhibitors during histone isolation, including a protease inhibitor cocktail (Roche), which inhibits enzymatic cleavage of H3 in human ESCs [26], and E-64, which inhibits cathepsins, including Cathepsin L, which cleaves H3 in mouse ESCs [11]. The H3 cleavage product that we observe in human PBMCs is similar in size to the H3 cleavage product observed in mouse ESCs [11]. Extensive H3 cleavage is observed in approximately one-third of these PBMC histone samples (**Figure 2**). {We did not find that age, sex, arsenic exposure, nutritional status, cigarette smoking status, or most of the other participant characteristics examined, were associated with extensive H3 cleavage (See **Appendix**, **Tables A1** and **A2**). However, H3 cleavage was significantly more common among individuals who had ever chewed betel nut (See Appendix, Table A2), a stimulant used in many parts of Asia, which has been classified as a human carcinogen by the International Agency for Research on Cancer [29]}[1].

Based on Western blot, we have determined that H3 cleavage interferes with the measurement of certain PTHMs. Figure 1A illustrates the known enzymatic cleavage sites in H3 for mouse ESCs [19]. In Figure 1B, Western blots illustrate total H3 (top panel) with varying degrees of histone cleavage for 11 representative PBMC histone samples that were collected from participants enrolled in the Folic Acid and Creatine Trial (FACT), a randomized controlled trial of folic acid and creatine supplementation in Bangladeshi adults [28]. Figure 1B also shows, for the same 11 PBMC samples, three PTHMs that vary in relation to their location on histone H3 (i.e., upstream or downstream of the cleavage sites shown in Figure 1A). For example,

{}[1] Indicates that this information has been added post-publication, because the referenced data was not available at the time of submission

Figure 1B illustrates H3K9me2 (second panel), a modification located downstream of known H3 cleavage sites. Samples without large amounts of H3 cleavage (Lanes 1, 3–5, 8, 10, 11) have detectable H3K9me2. In contrast, samples with extensive cleavage of H3 (Lanes 2, 6, 7, 9) have no detectable H3K9me2. Figure 1B also illustrates H3K36me2 (third panel) and H3K79me2 (fourth panel), which are PTHMs located upstream of H3 enzymatic cleavage sites; H3K36me2 is located in the tail region of H3, and H3K79me2 is located in the core domain of H3 (Figure 1A). H3K36me2 and H3K79me2 can be detected both in the 17-kDa band of H3 that has not been cleaved and in the <17-kDa bands of H3 that have been cleaved (Figures 1B and 2). H3 cleavage is also detectable in histones from calf thymus (Figure 2). This has been described previously by other groups [3, 30]. Similarly, cleavage of calf thymus H3 does not interfere with upstream PTHMs, such as H3K79me2 (Figure 2). Collectively, these data suggest that H3 cleavage only influences the ability to detect PTHMs that are situated downstream of histone cleavage sites.

{Although we determined that the ability to detect H3K36me2 and H3K79me2 is not impacted by H3 cleavage, global levels of these PTHMs were found to be significantly higher among participants with evident H3 cleavage (Table A1). This finding for H3K36me2 is consistent with a recent study by Tvardovskiy et al., which identified H3 cleavage in primary human hepatocytes [31]. Tvardovskiy et al. compared PTHM profiles in the N-terminal tails of intact vs. clipped histone H3 and observed that the clipped proteoform was enriched with H3K36me2 [31]}[1].

{}[1]Indicates that this information has been added post-publication, because the referenced data and publication were not available at the time of submission

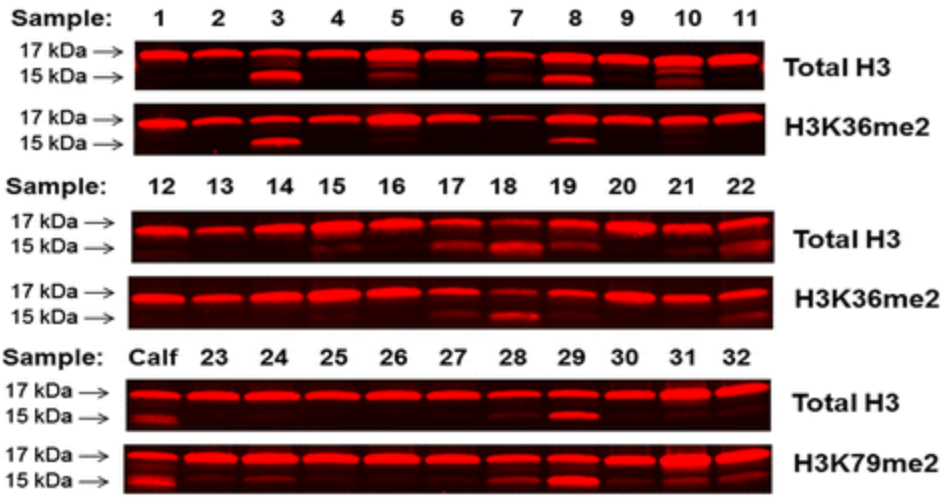

Figure 2. Extensive H3 cleavage is evident in approximately one-third of PBMC histone samples, but it does not affect measures of H3K36me2 and H3K79me2. Total H3 was measured in an additional 32 histone PBMC samples from the FACT study and in histones from calf thymus (Sigma-Aldrich). H3K36me2 was also measured in 22 of the PBMC samples (Samples 1–22), and H3K79me2 was measured in calf histones and in ten of the PBMC samples (Samples 23–32). Abbreviations used: FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; PBMC, peripheral blood mononuclear cell

IMPLICATIONS FOR MOLECULAR EPIDEMIOLOGY STUDIES:

Since it is unclear when enzymatic cleavage of H3 occurs in human PBMC samples, it is difficult to know if it is of biological or methodological interest. Regardless, enzymatic cleavage of H3 has important implications for measuring global PTHMs in human samples. Currently, the most commonly studied PTHMs include methylation and acetylation marks on H3K4, H3K9, and H3K27, which all fall within the N-terminal tail region of H3. However, the portion of H3 that is clipped off in mouse and human cells includes these residues. Thus, measures of marks on H3K4, H3K9, and H3K27 may be underestimated if samples have experienced enzymatic cleavage of H3. This is particularly true for antibody-based methods, such as ELISA and immunohistochemistry methods, which cannot take into account H3 cleavage. PTHMs that lie on

amino acid residues upstream of H3 enzymatic cleavage sites, such as H3K36 and H3K79, do not appear to be affected by the enzymatic cleavage of H3 and therefore can be measured accurately, regardless of cleavage.

A better understanding of when and why enzymatic cleavage of H3 occurs is essential. If enzymatic cleavage of human H3 occurs *in vivo*, this may be an important biological phenomenon. Alternatively, if enzymatic cleavage occurs as a result of sample collection and processing, preventive measures must be developed such that all PTHMs on H3, including modifications on H3K4, H3K9, and H3K27, can be accurately measured. In the meantime, for banked samples previously collected for the measurement of global PTHMs, Western blot can be used to check samples for enzymatic cleavage of histones. If histone cleavage products are observed in samples, it may not be appropriate to measure certain PTHMs based on their location.

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CHAPTER FOUR APPENDIX

Table A1. Descriptive characteristics[a] for continuous variables in FACT participants with vs. without extensive H3 cleavage

	Extensive H3 Cleavage	**Minimal/No H3 Cleavage**	***P*[b]**
H3K36me2 (% of total H3)	1.56 (1.03-6.87), *n* = 42	1.35 (0.84-3.13), *n* = 73	<0.01
H3K36me3 (% of total H3)	1.66 (0.80-6.00), *n* = 41	1.44 (0.48-3.72), *n* =70	0.10
H3K79me2 (% of total H3)	1.55 (0.50-9.41), *n* = 42	1.26 (0.68-3.53), *n* = 73	0.03
Age (y)	40 (27-52), *n* = 42	38 (24-54), *n* = 73	0.10
BMI (kg/m^2)	18.6 (15.3-26.9), *n* = 41	19.1 (15.7-28.0), *n* = 68	0.80
Education (y)	0.5 (0.0-10.0), *n* = 42	3 (0-12), *n* = 73	0.13
RBC Folate (nmol/L)	469 (186-825), *n* = 30	481 (155-1347), *n* = 56	0.85
Plasma Folate (nmol/L)	13.4 (4.2-54.7), *n* = 42	11.8 (4.1-38.3), *n* = 73	0.28
Plasma Cobalamin (pmol/L)	222 (60-705), *n* = 42	201 (78-610), *n* = 73	0.25
Plasma Choline (µmol/L)	10.7 (8.1-18.1), *n* = 42	11.4 (7.3-20.0), *n* = 73	0.38
Plasma Betaine (µmol/L)	43.9 (22.8-85.6), *n* = 42	42.3 (18.3-86.4), *n* =73	0.49
Plasma Hcys (µmol/L)	10 (6-24), *n* = 42	13 (4-102), *n* = 73	0.15
Cys (µmol/L)	223 (147-370), *n* = 41	230 (137-326), *n* = 71	0.15
uCr (µg/dL)	41 (9-198), *n* = 42	52 (6-233), *n* = 73	0.34
uAs (µg/L)	108 (17-958), *n* = 42	137 (11-1767), *n* = 73	0.52
uAs_{Cr} (µg/g)	256 (48-975), *n* = 42	218 (65-2203), *n* = 73	0.60
bAs (µg/L)	9.0 (2.2-26.9), *n* = 42	8.4 (2.4-80.2), *n* = 73	0.97
bSe (µg/L)	134 (92-186), *n* = 42	132 (91-167), n = 73	0.73

Abbreviations used: BMI, body mass index; bSe, blood selenium; Cys, cysteine; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; Hcys, homocysteine; RBC, red blood cell; uAs, urinary arsenic; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine

[a]Median (range) values of continuous characteristics of FACT participants with vs. without extensive H3 cleavage, identified by Western blot. The methods used for measuring these characteristics are described in Chapters 5 and 7.
[b]Two-sided Wilcoxon rank-sum test

Table A2. Descriptive characteristics[a] for categorical variables in FACT participants with vs. without H3 cleavage

	Extensive H3 Cleavage	**Minimal/No H3 Cleavage**	***P*[b]**
Sex			
Males (*n* = 58)	20 (34.5)	38 (65.5)	0.65
Females (*n* = 57)	22 (38.6)	35 (61.4)	
Betel Nut Status			
Ever Betel (*n* = 34)	19 (55.9)	15 (44.1)	<0.01
Never Betel (*n* = 80)	23 (28.8)	57 (71.2)	
Cigarette Smoking Status			
Ever smoked cigarette (*n* = 33)	13 (39.4)	20 (60.6)	0.72
Never smoked cigarette (*n* = 81)	29 (35.8)	52 (64.2)	
TV Ownership			
Own TV (*n* = 40)	13 (32.5)	27 (67.5)	0.51
No TV (*n* = 75)	29 (38.7)	46 (61.3)	
Land Ownership			
Own Land (*n* = 58)	19 (32.8)	39 (67.2)	0.40
No Land (*n* = 57)	23 (40.4)	34 (59.6)	
Folate Status			
Plasma Folate <9 nmol/L (*n* = 23)	7 (30.4)	16 (69.6)	0.50
Plasma Folate ≥9 nmol/L (*n* = 92)	35 (38.0)	57 (62.0)	
Cobalamin Status			
Plasma Cobalamin <151 pmol/L (*n* = 30)	8 (26.7)	22 (73.3)	0.19
Plasma Cobalamin ≥151 pmol/L (*n* = 85)	34 (40.0)	51 (60.0)	
Hyperhomocysteinemia			
Plasma Hcys >13 μmol/L (*n* = 47)	13 (27.7)	34 (72.3)	0.10
Plasma Hcys ≤13 μmol/L (*n* = 68)	29 (42.6)	39 (57.4)	

Abbreviations used: FACT, Folic Acid and Creatine Trial; Hcys, homocysteine

[a]*n* (%) of participants in specified subgroup with or without H3 cleavage. The methods used for measuring these characteristics are described in Chapters 5 and 7.
[b]Chi-square test

CHAPTER FIVE

Associations between blood and urine arsenic concentrations and global levels of posttranslational histone modifications in Bangladeshi men and women

Caitlin G. Howe[1], Xinhua Liu[2], Megan N. Hall[3], Vesna Slavkovich[1], Vesna Ilievski[1], Faruque Parvez[1], Abu B. Siddique[4], Hasan Shahriar[4], Mohammad N. Uddin[4], Tariqul Islam[4], Joseph H. Graziano[1], Max Costa[5], Mary V. Gamble[1]

Affiliations: Departments of [1]Environmental Health Sciences, [2]Biostatistics, and [3]Epidemiology, Mailman School of Public Health, Columbia University, New York, New York, USA; [4]Columbia University Arsenic Project in Bangladesh, Dhaka, Bangladesh; and the [5]Department of Environmental Medicine, Langone Medical Center, New York University, New York, New York, USA

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ABSTRACT

Background: Exposure to inorganic arsenic is associated with numerous adverse health outcomes, with susceptibility differing by sex. While evidence from *in vitro* studies suggests that arsenic alters posttranslational histone modifications (PTHMs), evidence in humans is limited.

Objectives: The objectives were to determine: 1) if arsenic exposure is associated with global levels of PTHMs: H3K36me2, H3K36me3, and H3K79me2 in a sex-dependent manner and 2) if PTHMs are stable when arsenic exposure is reduced.

Methods: We examined associations between arsenic, measured in blood and urine, and PTHMs in peripheral blood mononuclear cells from 317 participants enrolled in the Bangladesh Folic Acid and Creatine Trial (FACT). We also examined the stability of PTHMs after the use of arsenic-removal water filters (n = 60).

Results: Associations between natural log-transformed (log) urinary arsenic, adjusted for creatinine (uAs_{Cr}), and H3K36me2 differed significantly between men and women (P = 0.01). Log(uAs_{Cr}) was positively associated with H3K36me2 in men (β: 0.12; 95% CI: 0.01, 0.23; P = 0.03), but was negatively associated with H3K36me2 in women (β: -0.05; 95% CI: -0.12, 0.02; P = 0.19). The patterns of associations with blood arsenic were similar. On average, water filter use was also associated with reductions in H3K36me2 (P < 0.01), but this did not differ significantly by sex. Arsenic was not significantly associated with H3K36me3 or H3K79me2 in men or women.

Conclusions: Arsenic exposure was associated with H3K36me2 in a sex-specific manner, but was not associated with H3K36me3 or H3K79me2. Additional studies are needed to assess changes in H3K36me2 after arsenic removal.

INTRODUCTION

Worldwide, more than 140 million people are exposed to arsenic-contaminated drinking water [1]; in Bangladesh alone, up to 57 million individuals are exposed [2]. Chronic exposure to arsenic causes bladder, lung, and skin cancers and is also associated with numerous non-cancer health outcomes [3]. Susceptibility to many of these arsenic-related health outcomes differs by sex, with some outcomes preferentially afflicting males and others females [3]. For example, males are more likely to develop arsenic-induced skin lesions [4,5] and skin, liver, and bladder cancers [6-8], while females may be more susceptible to arsenic-induced developmental outcomes [9-11] and cardiovascular disease [12]. However, the mechanisms underlying these sex differences remain unknown.

Experimental studies and observational studies in human populations have demonstrated that arsenic alters epigenetic modifications, including global 5-methylcytosine (5-mC) [13-16] and 5-hydroxymethylcytosine (5-hmC) [17,18], and there is evidence that these effects differ by sex [17, 19-21]. *In vitro* and rodent studies have also shown that arsenic alters global levels of posttranslational histone modifications (PTHMs) in tissues or cell lines derived from tissues that are targets of arsenic toxicity, such as the lung [22], bladder [23], and brain [24], and the effects of arsenic on PTHMs in the brain have been shown to be sex-dependent in mice [25]. An epidemiological study of 63 male steel workers also reported that arsenic exposure via inhalation was associated with higher global levels of histone H3 lysine 4 dimethylation in white blood cells (WBCs) [26]; however, since this study only included men, potential differences by sex could not be examined.

In a previous study of 40 Bangladeshi adults, we observed sex-specific associations between arsenic, measured in urine, and several PTHMs (H3 lysine 4 trimethylation, H3 lysine 27 trimethylation, and H3 lysine 27 acetylation) in peripheral blood mononuclear cells (PBMCs) [27]. We have also previously observed sex-specific associations between arsenic exposure and global levels of both 5-mC and 5-hmC in PBMC DNA [17]. Thus, we now present data on three PTHMs: histone H3 lysine 79 di-methylation (H3K79me2), selected because it has been shown to regulate the expression of Tet1 [28,29], which converts 5-mC to 5-hmC [30], and it is dysregulated in cancers [31,32], and histone H3 lysine 36 di- and tri-methylation (H3K36me2 and H3K36me3, respectively), because these two PTHMs have been shown to be altered by arsenic *in vitro* [22] and they are also dysregulated in several types of cancer [33-36]. This study utilized a subset of PBMC samples collected from participants enrolled in the Folic Acid and Creatine Trial (FACT) [37]. First we evaluated sex-specific associations between arsenic and our candidate PTHMs using baseline FACT samples. Then, since all participants in the trial were provided with arsenic-removal water filters at enrollment, we evaluated whether PTHMs were altered after reducing arsenic exposure; this was achieved using samples collected at baseline and week 12 from participants who did not receive a dietary supplement. The data reported herein add to a growing body of evidence that arsenic induces epigenetic dysregulation on a global level and, moreover, that this often occurs in a sex-specific manner.

STUDY PARTICIPANTS AND METHODS

Region and Participants

In 2010, participants for the current study (FACT) were recruited from the Health Effects of Arsenic Longitudinal Study (HEALS), a prospective cohort study which initially recruited 11,746 adults (between the ages of 20 and 65) living in a 25 km^2 region in Araihazar,

Bangladesh [38]. FACT participants were randomly selected from all HEALS participants who had been drinking from household wells with water arsenic ≥50 μg/L, the Bangladesh standard for safe drinking water. Exclusion criteria included: pregnancy, nutritional supplement use, and adverse health outcomes, including proteinuria, renal disease, diabetes, gastrointestinal disease, chronic obstructive pulmonary disease, skin lesions, and cancer. Informed consent was obtained by Bangladeshi field staff physicians. This study was approved by the Institutional Review Board of Columbia University Medical Center and the Bangladesh Medical Research Council.

Study Design

The FACT study is a randomized, placebo-controlled trial that had the primary goal of determining whether folic acid (FA) and/or creatine supplementation reduces blood arsenic (bAs) concentrations in arsenic-exposed, Bangladeshi adults [37]. All FACT participants received an arsenic-removal water filter (READ-F filter, Brota Services International, Bangladesh) at baseline to be used for the duration of the study and thereafter. Participants (n = 622) were also randomized to one of five nutrition intervention treatment arms: placebo (n =104), 400 μg FA/day (n = 156), 800 μg FA/day (n = 154), 3 g creatine/day (n = 104), and 3 g creatine + 400 μg FA/day (n = 104) [37]. Whole blood and urine samples were collected from participants at baseline, week 12, and week 24; sample collection and handling have been described previously [27,37]. For the current study, we used histones isolated from baseline (i.e., pre-intervention)-collected PBMCs from a subset of FACT participants from all five treatment arms (see **Supplemental Material, Figure S1**), who had whole blood, urine, and PBMC samples and complete data for arsenic measures, PTHMs, and potential confounders (n = 317). We also used all available PBMCs collected at baseline and week 12 from participants in the placebo group (n = 60) to examine if PTHMs were altered after the use of arsenic-removal water

filters; due to filter use, participants in the placebo group experienced a significant decrease in bAs concentrations from baseline to week 12 [37].

General Characteristics

General characteristics of the study participants were determined at baseline by an in-person questionnaire. Body mass index (BMI) was calculated from the measured weight and height of each participant (kg/m^2) at baseline.

Total Blood Arsenic

As described previously [37], total bAs concentrations were measured using a Perkin-Elmer Elan DRC II ICP-MS equipped with an AS10+ autosampler. The intra- and inter-assay CVs for bAs were 2.7% and 5.7%, respectively.

Total Urinary Arsenic

We measured total urinary arsenic (uAs) by graphite furnace atomic absorption spectrophotometry, using the AAnalyst 600 graphite furnace system (Perkin Elmer, Shelton, CT), based on a method by Nixon et al. [39]. Intra- and inter-assay CVs for uAs were 3.1% and 5.4%, respectively. These values were adjusted for urinary creatinine (uCr) concentrations, measured by a method based on the Jaffe reaction [40]. The intra- and inter-assay CVs for uCr were 1.3% and 2.9%, respectively.

Plasma Folate and Cobalamin

Plasma folate and cobalamin were measured by radio-protein-binding assay (SimulTRAC-SNB, MP Biomedicals). The intra- and inter-assay CVs were 5% and 13%, respectively, for folate and 6% and 17%, respectively for cobalamin.

Histone Isolation

While we recently identified a cleavage product of histone H3 in human PBMCs that interferes with the measurement of PTHMs residing downstream of H3 cleavage sites [41], we note that the PTHMs in the current study are located upstream of cleavage sites and are therefore not impacted by this. Histones were isolated from PBMCs by acid extraction, as described previously [27]. Briefly, PBMCs were lysed in radioimmunoprecipitation assay buffer, supplemented with a protease inhibitor cocktail (Roche) and 1 µM of protease inhibitor E-64, for 10 min. The cell lysate was passed through a 21-gauge needle, and the pellet was collected by centrifugation, washed in histone washing buffer, collected again after centrifugation, and resuspended in 0.4 N H_2SO_4. After incubation at 4°C overnight, the supernatant was collected by centrifugation, mixed with acetone, and incubated overnight at -20°C. Pellets were collected by centrifugation, washed with acetone, dried, and resuspended in 4 M urea. Histone concentrations were determined by the Bradford Assay, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Samples were aliquoted and stored at -80°C.

H3K36me2, H3K36me3, H3K79me2

PTHMs were measured by sandwich ELISA, based on a previously described method [27]. Polystyrene 96-well microplates (Fisher Scientific) were coated with a capture antibody for total histone H3 (Abcam, 1:20,000) and incubated overnight at 4ºC. The next day, plates were blocked with 3% milk diluted in PBST (1 X PBS, 0.05% TWEEN-20) for 2 hr and then washed with PBST. Histone samples from FACT participants were diluted with ddH_2O. Sample dilutions for each assay were as follows: H3K36me2, 1 ng/µL; H3K36me3, 1.5 ng/µL; H3K79me2, 2.0 ng/µL. A standard curve was made with mixed histones from calf thymus (Sigma), and a pooled blood sample was included on each plate for calculating inter-assay CVs. FACT histone samples,

calf histones, and the pooled blood sample were plated in duplicate. Plates were incubated on an orbital shaker at room temperature for 1.5 hr, then wells were washed with PBST. Detection antibodies were diluted in 1% milk PBST, to further prevent potential background signal, and 100 µL was added to each well of the appropriate plate. Detection antibody dilutions were as follows: total H3, Sigma, 1:40,000; H3K36me2, Abcam, 1:2,000; H3K36me3, Abcam, 1:2,000; H3K79me2, Active Motif, 1:1,000. Plates were incubated for 1 hr at room temperature on an orbital shaker. Plates were washed with TBST (0.1% TWEEN-20), and 100 µL of secondary antibody (Santa Cruz, goat anti-rabbit IgG-HRP, 1:2000, diluted in TBS) was added to each well. Plates were incubated for 1 hr at room temperature without agitation. Subsequently, plates were washed with TBST followed by ddH_2O, and 100 µL of 3,3',5,5'-tetramethylbenzidine was added to each well. Plates were incubated in the dark for 10 min. The reaction was quenched with 2 N H_2SO_4, and the optical density was read at 450 nm using a SpectraMax 190 plate reader (Molecular Devices) with SoftMax Pro software (version 6.3). Total H3 and each of the three PTHMs were calculated relative to mixed calf histones based on a 4-parameter logistic standard curve. The % PTHM level was calculated by dividing the PTHM measure by the total H3 measure. Samples from the same individual, but from different time points, were run on the same plate. H3K79me2 values were normalized to the pooled blood sample to reduce potential batch effects [42]. The inter-assay CV for H3K79me2 was calculated from a subset of samples (n = 16) measured on two separate days. The intra- and inter-assay CVs, respectively, for each ELISA method were as follows: H3K36me2: 3.4% and 9.6%, H3K36me3: 4.9% and 11.9%, and H3K79me2: 7.1% and 7.0%. Since there were limited histone aliquots for the final assays, and since samples with poor reproducibility were excluded, final sample sizes for H3K36me2 (n = 311) and H3K36me3 (n = 300) were smaller than the final sample size for H3K79me2 (n = 315).

Statistical Methods

Summary statistics were calculated for each variable (median (range) for continuous variables and % for categorical variables) in all participants and also separately by sex. Differences in continuous and categorical variables, between men and women and also between participants with and without PTHM measures, were determined by Wilcoxon rank-sum and Chi-square tests, respectively. Transformations were applied to variables with skewed distributions to stabilize variances for parametric model assumptions and to reduce the influence of extreme values. A natural log-transformation (log(X)) was applied to each of the predictors, bAs and uAs, which was adjusted for uCr (uAs_{Cr}); to the covariate BMI; and also to two of the outcome variables, H3K36me3, H3K79me2. An inverse transformation (1/Y) was applied to the third outcome variable, H3K36me2.

A generalized linear model, with an inverse-link function applied to the mean of (1/Y), was used to model the association between log(bAs) or log(uAs_{Cr}) and the harmonic mean of H3K36me2. Associations between the predictors, log(bAs) and log(uAs_{Cr}) and the outcomes, log(H3K36me3) and log(H3K79me2), were examined with linear models. Arsenic regression coefficients (β) in models for H3K36me2 indicate the change in the harmonic mean of H3K36me2 for a unit increase in log(bAs) or log(uAs_{Cr}), controlling for other variables in the model, while those for H3K36me3 and H3K79me2 indicate the change in the mean of the log(PTHM) for a unit increase in log(bAs) or log(uAs_{Cr}), controlling for other variables in the model. Variables were considered potential confounders if they were correlated with arsenic exposure measures and the PTHM in men or women and their addition to models changed arsenic exposure coefficients by >10%. Therefore, we also present models adjusted for age, log(BMI), education, and sex. To demonstrate the robustness of the associations between arsenic

measures and PTHMs, we also present analyses in supplemental materials showing adjustment for additional variables: log(uCr), log(plasma folate), and log(plasma cobalamin), and also cigarette and betel nut use (ever vs. never). All variables were included in models as continuous variables, except for sex and education; the latter was dichotomized (education >5 years vs. ≤5 years), since many participants had 0 years of education. Models were also run separately by sex, and differences by sex were determined using the Wald test, which compares regression coefficients between models [43].

We also present Spearman correlations, which remain the same with or without applying the specified transformations to variables, showing the relationships between arsenic measures and PTHMs to confirm that the directions of the associations are consistent with model-based results and to facilitate comparisons between PTHMs.

Relationships between baseline and week 12 measures of each PTHM were examined using Spearman correlations. The Wilcoxon signed-rank test was used to evaluate whether PTHMs (untransformed) changed on average over a 12 week period; this was examined in a subset of participants (n = 60) in the placebo group (n = 56 for H3K36me2 and H3K79me2, n = 55 for H3K36me3). We also examined the within-person changes in the PTHMs separately by sex and tested for differences using the Wilcoxon rank-sum test.

A significance level of 0.05 was used for all statistical tests and regression models, which were performed with SAS (version 9.3, Cary, NC).

RESULTS

General Characteristics, Arsenic Measures, and PTHMs

General characteristics for the study participants are presented in **Table 1**. Participants were between 24 and 54 years old. Approximately 22.4% of the study participants had >5 years of education. Blood arsenic concentrations ranged from 1.0 to 80.2 μg/L. Concentrations of uAs_{Cr} ranged from 35 to 2200 μg/g uCr. Compared with women, male study participants were older, had lower BMIs, and had higher uCr and bAs concentrations and lower uAs_{Cr} concentrations. Men were also more likely to have low plasma folate concentrations and to be cigarette smokers. Individuals in the placebo group with PTHM measures (See **Supplemental Material, Table S1**) were very similar to the overall study population (Table 1). The only variables which differed were uCr concentrations and, consequently, uAs concentrations, which were both lower in the placebo group participants. However, uAs_{Cr} concentrations were similar between groups.

FACT participants with PTHM measures were generally comparable to FACT participants without PTHM measures (See **Supplemental Material, Table S2**), although participants with PTHM measures were slightly older, were more likely to have low folate, and had higher uCr and uAs concentrations (before adjustment for uCr).

Associations between Arsenic Exposure and PTHMs

In the whole sample, neither log(bAs) nor $\log(uAs_{Cr})$ was significantly associated with any of the PTHMs (**Table 2**). However, $\log(uAs_{Cr})$ was positively associated with H3K36me2 in men both before (β: 0.13; 95% CI: 0.02, 0.24; $P = 0.02$) and after (β: 0.12; 95% CI: 0.01, 0.23; $P = 0.03$) adjusting for age, education, and log(BMI). The association between $\log(uAs_{Cr})$ and

Table 1. Baseline characteristics[a] of FACT participants with at least one PTHM measure and complete information for variables included in regression models

Characteristic	Whole Sample (317)	Men (161)	Women (156)	*P*[b]
Age (y)	39 (24-54)	42 (25-54)	37 (24-54)	<0.01
BMI (kg/m^2)	19.3 (13.9-31.6)	18.7 (15.4-27.9)	20.0 (13.9-31.6)	<0.01
uCr (μg/L)	48 (6-252)	53 (6-252)	45 (6-233)	0.03
bAs (μg/L)	8.8 (1.0-80.2)	9.6 (2.5-52.0)	7.9 (1.0-80.2)	0.05
uAs (μg/L)	121 (11-1770)	123 (11-1770)	121 (11-1320)	0.67
uAs_{Cr} (μg/g uCr)	257 (35-2200)	242 (65-1480)	287 (35-2200)	0.03
H3K36me2[c] (% of total H3)	1.45 (0.68-6.87)	1.45 (0.68-4.00)	1.43 (1.00-6.87)	0.47
H3K36me3[d] (% of total H3)	1.61 (0.48-6.44)	1.57 (0.48-4.09)	1.62 (0.52-6.44)	0.18
H3K79me2[e] (% of total H3)	1.26 (0.29-9.46)	1.26 (0.29-9.46)	1.25 (0.29-9.41)	0.69
Folate <9 nmol/L	74 (23.3)	46 (28.6)	28 (18.0)	0.03
Cobalamin <151 pmol/L	77 (24.3)	39 (24.2)	38 (24.4)	0.98
Ever Smoker	93 (29.3)	91 (56.5)	2 (1.3)	<0.01
Ever Betel	87 (27.4)	48 (29.8)	39 (25.0)	0.34
Education >5 y	77 (22.4)	33 (20.5)	38 (24.4)	0.41

Abbreviations used: bAs, blood arsenic; BMI, body mass index; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; PTHM, posttranslational histone modification; uAs, urinary arsenic; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine; uCr, urinary creatinine

[a]Values are median (range) or *n* (%) for continuous and categorical variables, respectively
[b]Wilcoxon rank-sum or Chi-square test for difference between men and women in continuous and categorical variables, respectively
[c]*n* = 311 (Men: 158, Women: 153)
[d]*n* = 300 (Men: 153, Women: 147)
[e]*n* = 315 (Men: 161, Women: 154)

H3K36me2 was in the opposite direction for women (covariate-adjusted β: -0.05; 95% CI: -0.12, 0.02; $P = 0.19$) and differed significantly from the corresponding estimate in men ($P = 0.01$) (Table 2). While not statistically significant, associations between log(bAs) and H3K36me2 were similar to those for log(uAs_{Cr}), with estimates that were positive in men and negative in women (P for difference between men and women = 0.08 for covariate-adjusted models). The patterns of associations according to sex were similar for log(H3K36me3). Since coefficients in models for H3K36me2 represent changes in the harmonic mean of H3K36me2, while coefficients in models for H3K36me3 represent changes in the mean of log(H3K36me3), the magnitudes of the associations cannot be directly compared. However, the findings were consistent when examined by Spearman correlation, which does not require that variables be transformed and thus allows for more direct comparisons between PTHMs (See **Supplemental Material, Table S3**). Although associations between log-transformed arsenic measures and log(H3K36me3) were not significant in either men or women, differences by sex were significant or suggestive (Table 2). Log-transformed arsenic measures were not associated with log(H3K79me2) in men or women, and differences by sex were not significant (Table 2). Associations between arsenic measures and PTHMs were very similar after additionally adjusting for log(uCr), log(plasma folate), log(plasma cobalamin), cigarette smoking status, and betel nut use (See **Supplemental Material, Table S4**).

Stability of PTHMs after Reductions in Arsenic Exposure

Arsenic-removal water filter use for 12 weeks was associated with significant reductions in bAs (see [37]) and uAs_{Cr} ($P < 0.01$, Wilcoxon signed-rank test) in the placebo group. Summary statistics for within-person changes in H3K36me2, H3K36me3, and H3K79me2

Table 2. Estimated regression coefficients[a] and 95% confidence intervals for associations between baseline measures of arsenic exposure and PTHMs in FACT participants

PTHM	Arsenic Exposure	Whole Sample	Men	Women	*P*[b]
H3K36me2[c]	bAs	0.02 (-0.05, 0.09)	0.12 (-0.00, 0.24)	-0.04 (-0.12, 0.04)	0.04
	bAs[d]	0.02 (-0.05, 0.09)	0.10 (-0.02, 0.22)	-0.03 (-0.11, 0.05)	0.08
	uAs_{Cr}	0.01 (-0.05, 0.08)	0.13 (0.02, 0.24)*	-0.05 (-0.12, 0.02)	<0.01
	uAs_{Cr}[d]	0.02 (-0.05, 0.08)	0.12 (0.01, 0.23)*	-0.05 (-0.12, 0.02)	0.01
H3K36me3[e]	bAs	-0.03 (-0.10, 0.04)	0.06 (-0.04, 0.16)	-0.08 (-0.17, 0.01)	0.04
	bAs[d]	-0.02 (-0.09, 0.05)	0.05 (-0.04, 0.15)	-0.07 (-0.16, 0.02)	0.07
	uAs_{Cr}	-0.01 (-0.07, 0.05)	0.07 (-0.02, 0.16)	-0.06 (-0.14, 0.02)	0.04
	uAs_{Cr}[d]	0.00 (-0.06, 0.06)	0.07 (-0.02, 0.16)	-0.05 (-0.14, 0.03)	0.05
H3K79me2[f]	bAs	0.04 (-0.05, 0.12)	0.04 (-0.09, 0.17)	0.03 (-0.08, 0.14)	0.91
	bAs[d]	0.03 (-0.05, 0.12)	0.04 (-0.09, 0.17)	0.04 (-0.08, 0.15)	0.95
	uAs_{Cr}	0.02 (-0.06, 0.09)	0.05 (-0.07, 0.17)	0.00 (-0.10, 0.10)	0.53
	uAs_{Cr}[d]	0.01 (-0.06, 0.09)	0.04 (-0.08, 0.17)	0.01 (-0.10, 0.11)	0.65

Abbreviations used: bAs, blood arsenic; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; PTHM, posttranslational histone modification; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine

[a]Estimated regression coefficients and 95% confidence intervals (β (CI)) from generalized linear models. Associations were examined between log(bAs) or log(uAs_{Cr}) in relation to each of the three PTHMs. Coefficients from H3K36me2 models indicate the change in the harmonic mean of H3K36me2 for a unit increase in the log-transformed arsenic measure, controlling for other covariates. Coefficients from H3K36me3 and H3K79me2 models indicate the change in the mean of the log(PTHM) for a unit increase in the log-transformed arsenic measure, controlling for other covariates.
[b]Wald test for sex difference
[c]Whole sample n = 311, Men n = 158, Women n = 153.
[d]Adjusted for age, education (dichotomized at 5 years), and log(BMI). Whole sample analyses were also adjusted for sex.
[e]Whole sample n = 300, Men n = 153, Women n = 147.
[f]Whole sample n = 315, Men n = 161, Women n = 154.
*$P < 0.05$

from baseline to week 12 for participants in the placebo group are presented in **Table 3**. Although for each of the three PTHMs analyzed, baseline values were significantly correlated with values measured at week 12 (*P*-values from Spearman correlations < 0.01), the median change in H3K36me2 from baseline to week 12 was negative (-0.15). Thus, on average H3K36me2 declined over time ($P < 0.01$), although the interquartile range (IQR) for the within-person change (-0.43, 0.11) indicates that this mark did increase over time in at least 25% of participants. In sex-stratified analyses, H3K36me2 was found to decrease on average among both men and women. However, the decline was only statistically significant among women ($P < 0.01$). H3K36me3 did not change significantly during the 12 week period, but there was a suggestive decrease in H3K79me2 (median within-person change: -0.05; IQR: -0.24, 0.04), $P = 0.07$). The within-person changes in PTHMs did not differ significantly by sex (Table 3).

DISCUSSION

In an adult population in Bangladesh, we examined associations between arsenic exposure and three PTHMs (H3K36me2, H3K36me3, and H3K79me2), which were selected because they are dysregulated in several types of cancer [31-36] and are altered by arsenic *in vitro* [22] or regulate 5-hmC [28,29]. Percent 5-hmC has been shown to be altered by arsenic in male rats [18] and in humans in a sex-dependent manner [17]. We observed that arsenic was associated with higher levels of H3K36me2, but only in men. Interestingly, the use of arsenic-removal water filters, which was associated with significant reductions in both bAs [37] and uAs_{Cr}, was also associated with significant reductions in H3K36me2 in the full sample. However, in sex-stratified analyses, while we observed that H3K36me2 declined in both men and women, this only achieved statistical significance among women. Given that there was no

Table 3. Within-person changes in PTHMs from baseline to week 12 for FACT participants in the placebo group

PTHM	Whole Sample			Men			Women			*p*[a]
	n	Median (IQR)	*P*[b]	*n*	Median (IQR)	*P*[b]	*n*	Median (IQR)	*P*[b]	
H3K36me2	56	-0.15 (-0.43, 0.11)	<0.01	27	-0.07 (-0.44, 0.16)	0.14	29	-0.17 (-0.37, 0.04)	<0.01	0.62
H3K36me3	55	0.02 (-0.23, 0.30)	0.61	28	0.05 (-0.23, 0.47)	0.35	27	0.02 (-0.22, 0.12)	0.93	0.35
H3K79me2	56	-0.05 (-0.24, 0.04)	0.07	29	-0.03 (-0.28, 0.09)	0.36	27	-0.05 (-0.18, 0.04)	0.10	0.88

Abbreviations used: FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; IQR, interquartile range; PTHM, posttranslational histone modification

[a]Wilcoxon rank-sum test for difference between men and women in the within-person change for each PTHM
[b]Wilcoxon signed-rank test for within-person change in PTHM from baseline to week 12

comparison group that did not receive arsenic-removal water filters due to ethical considerations, we cannot rule out the possibility that the decline in H3K36me2 may have been caused by extrinsic factors. Additional studies will be needed to confirm the changes we observed in H3K36me2 in association with arsenic removal. In cross-sectional analyses, arsenic exposure was not associated with H3K36me3. Additionally, H3K36me3 did not change over time, despite reductions in arsenic exposure. Thus, arsenic does not appear to alter H3K36me3 in histones derived from PBMCs. Although in cross-sectional analyses arsenic exposure was not associated with H3K79me2, which has been shown to regulate the expression of Tet1 [28,29], we did observe a suggestive ($P = 0.07$) decline in H3K79me2 over time after individuals received arsenic-removal water filters; this needs to be confirmed in a larger sample.

Although neither H3K36me3 nor H3K79me2 changed significantly over the 12 week period, we cannot make definitive conclusions about the stability of these marks, since all participants received arsenic-removal water filters at baseline and were thus subject to an intervention; furthermore, these marks did vary over time in some participants. Little is known about the stability of PTHMs in human PBMCs. One study by Zhang et al. examined the stability of PTHMs during adipogenesis and observed that while gene-specific levels of PTHMs were highly dynamic, global levels of PTHMs were remarkably stable [44]. However, since Zhang et al. used murine adipocyte cell lines, it is unclear if these findings are relevant to PTHM stability in human PBMCs. One previous epidemiological study measured PTHMs in PBMCs collected at three one-week intervals from 15 nickel refinery workers and 15 individuals who had not been exposed occupationally to nickel, and observed that the inter-individual variances in PTHMs were much higher than the intra-individual variances, suggesting that PTHMs are relatively

stable over time in human PBMCs [45]. However, since this was evaluated over a short duration, and in a small number of participants, this is an area that requires additional investigation.

Our study has several potential limitations. First, given the cross-sectional nature of some of the analyses, we need to consider the possibility of reverse causality. Since several previous experimental studies have shown that arsenic influences PTHMs, it is unlikely that reverse causality would explain our findings. However, it is possible that PTHMs influence the expression of genes involved in arsenic metabolism, such as the arsenic (+3 oxidation state) methyltransferase, which could thereby influence the excretion of arsenic, thus altering bAs and uAs_{Cr} concentrations. Although residual confounding is another important consideration, the associations between arsenic measures and PTHMs were quite robust, even after adjusting for additional covariates. Another important consideration for our study is the fact that PTHMs were measured in human PBMCs, which consists of a mixed population of cell types. However, global DNA methylation levels, which are closely related to PTHMs, have been shown to be very similar between blood cell types (reviewed in [46]). Additionally, a cross-sectional study of 63 male steel workers, which examined associations between inhalation exposure to occupational toxicants, including arsenic, and PTHMs in total WBCs, evaluated the influence of cell type distribution on these associations, and observed that while adjusting for the proportion of granulocytes influenced their results, adjusting for other cell types did not have a major impact [26]. Since we measured PTHMs in PBMCs, which do not include granulocytes, potential shifts in the proportion of granulocytes could not explain the associations we observed between arsenic and PTHMs. However, we cannot rule out the possibility that alterations in the proportion of monocytes, natural killer cells, T cells, or B cells, or their subpopulations, may have affected our findings, and this is an area that merits additional investigation. Another important limitation of

our study is that the sample sizes for prospective analyses were small. Therefore, we may have had insufficient statistical power to formally examine sex differences in the influence of arsenic removal on PTHMs. Finally, we recently identified a specific cleavage product of histone H3 in human PBMCs and observed that global levels of H3K36me2 and H3K79me2 were significantly higher in samples with extensive H3 cleavage ([41] and see Chapter 4). Therefore, while H3 cleavage does not impact the measurement of PTHMs, it is possible that this phenomenon nevertheless impacted our findings.

Despite some of the limitations of this study, our findings support a previous experimental study in A549 cells, which examined the effects of arsenite (2.5 and 5 μM) on H3K36me2 and H3K36me3 [22] and observed that arsenic decreased H3K36me2 and increased H3K36me3. Although we observed a positive association between uAs_{Cr} and H3K36me2 in men and did not observe a significant association between uAs_{Cr} and H3K36me3, we studied a population exposed to arsenic-contaminated drinking water for years to decades, while Zhou et al. measured PTHMs in a cell line that was exposed to arsenic for a 24 hour period. Furthermore, our study participants were healthy individuals, whereas A549 cells are alveolar basal epithelial cells derived from a male human lung tumor; arsenic may have distinct effects in different tissues and may also have differential effects in normal vs. cancerous cells. Additionally, *in vitro* studies are limited in that they cannot account for the numerous systemic differences associated with sex *in vivo*. Nevertheless, it is quite interesting that arsenic appears to target H3K36me2 in such diverse models.

Although the consequences of arsenic-induced increases in H3K36me2 are currently unknown, H3K36me2 has been implicated in oncogenic programming [47], and some of the enzymes responsible for regulating this mark, such as methyltransferase NSD2, are

overexpressed in multiple cancers, including those caused by arsenic, such as bladder, lung, and skin cancers [48]. A global increase in H3K36me2 leads to widespread increases in this mark across the genome, thereby altering its typical distribution [49]; this may have profound effects on both gene expression and genomic stability. For example, a global increase in H3K36me2 is associated with increased levels of H3K36me2 within gene bodies, which in turn is associated with increased expression of genes involved in oncogenic programming [47].

Similar to our findings, several studies have observed sex-specific effects of arsenic on other epigenetic marks, such as DNA methylation [19-21], including our previous finding that arsenic exposure is associated with increased global levels of 5-mC and 5-hmC in men but not women [17]. Sex-specific effects of other environmental contaminants, such as cadmium and lead, on DNA methylation have also been observed [50,51]. Since PTHMs can direct DNA methylation patterns [52], PTHMs may mediate the effects of environmental contaminants, such as arsenic, on DNA methylation marks. In addition to the sex-specific findings for uAs_{Cr} and H3K36me2 reported here, our group previously observed sex-specific correlations between uAs_{Cr} and several other PTHMs [27]. Additionally, Tyler et al. recently demonstrated that arsenic alters PTHMs in a sex-dependent manner in the mouse brain [25]. Although the mechanisms are not fully understood, arsenic also altered the expression of corresponding histone modifying enzymes, including MLL and KDM5B, in a sex-dependent manner [25]. Many histone modifying enzymes have also been shown to interact with androgen receptor [53], and some histone demethylases are dosage-sensitive regulators that are coded for by genes that reside on the Y chromosome and are highly conserved across mammalian species and broadly expressed across tissues and cell types [54]. Thus, both hormonal influences and genetic differences may contribute to the sex-specific effects of arsenic on PTHMs.

For many arsenic-induced health outcomes, susceptibility differs by sex [3]. For example, men are more susceptible to developing arsenic-induced skin lesions [4,5], and cancers of the skin, liver, and bladder [6-8]. In contrast, several studies have reported that early life exposure to arsenic is associated with impaired intellectual function and other developmental outcomes among female, but not male, children [9-11]. Additionally, in the United States the arsenic-associated risk for cardiovascular disease was found to be higher among women [12]. Animal studies have also demonstrated sex-specific effects of arsenic for many outcomes. For example, female mice are more susceptible to arsenic-induced changes in locomotor activity [55] and are more likely to develop lung tumors as a result of prenatal exposure to arsenic, while males are more likely to develop liver and adrenal tumors [56].

Although some of the sex-specific effects of arsenic observed in human populations may be explained by gender-differences in co-exposures (e.g., cigarette smoking, UV exposure, and nutritional deficiencies), the dramatic sex differences observed in well-controlled animal studies of arsenic toxicity suggest that co-exposures are not solely responsible for these differences. One consideration is that women have a higher capacity to fully methylate inorganic arsenic to dimethyl arsenic species, which facilitates arsenic elimination in urine [57-59]. This should generally reduce arsenic toxicity for women. However, some arsenic-related health outcomes preferentially afflict women, thus, there are likely other contributing factors. Epigenetic dysregulation has been implicated in the development of arsenic-induced health outcomes, including skin lesions and cancers of the skin and bladder [60-63]. Thus, epigenetic dysregulation may be one important mechanism contributing to the sex differences observed for multiple arsenic-related health outcomes. Previous studies examining the sex-specific effects of

arsenic on epigenetics have focused on DNA methylation. However, this study and our previous study [27] suggest that arsenic also induces sex-specific alterations in PTHMs.

CONCLUSIONS

Our findings have two major implications that warrant further investigation. First, arsenic exposure was associated with H3K36me2 in a sex-dependent manner in our study population of adults in Bangladesh. While it is tempting to speculate that these findings may explain some of the observed sex differences in susceptibility to arsenic-induced diseases, the impact of H3K36me2 and other PTHMs on health outcomes will require further study. Secondly, the arsenic-associated increase in H3K36me2 observed in men decreased, albeit non-significantly, after the use of arsenic-removal water filters. However, since we did not have a comparison group that did not receive water filters, and since H3K36me2 decreased significantly in women, future studies will be needed to evaluate the effects of arsenic removal on H3K36me2 and to investigate whether downstream effects of alterations in PTHMs, such as changes in DNA methylation patterns, persist over time.

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CHAPTER FIVE SUPPLEMENTAL MATERIAL

Table S1. Baseline characteristics[a] of FACT participants in the placebo group with PTHM measures

Characteristic	Whole Sample (*n* = 60)	Men (*n* = 31)	Women (*n* = 29)	*P*[b]
Age (y)	38 (25-53)	39 (25-53)	35 (27-52)	0.37
BMI (kg/m^2)[c]	19.5 (15.4-31.6)	18.7 (15.4-24.0)	21.3 (16.6-31.6)	<0.01
uCr (μg/L)	40 (6-121)	33 (6-121)	45 (8-102)	0.26
bAs (μg/L)	8.7 (1.0-34.7)	9.7 (3.9-17.8)	8.0 (1.0-34.7)	0.24
uAs (μg/L)	88 (11-796)	71 (11-305)	116 (22-796)	0.16
uAs_{Cr} (μg/g uCr)	297 (35-1250)	245 (91-598)	297 (35-1250)	0.33
H3K36me2[d] (% of total H3)	1.56 (0.68-3.86)	1.60 (0.68-3.86)	1.52 (1.03-2.33)	0.20
H3K36me3[e] (% of total H3)	1.67 (0.80-4.16)	1.73 (0.80-3.94)	1.66 (0.85-4.16)	0.87
H3K79me2[f] (% of total H3)	1.22 (0.66-3.46)	1.16 (0.29-2.78)	1.22 (0.66-3.46)	0.75
Folate <9 nmol/L	11 (18.3)	7 (22.6)	4 (13.8)	0.51[g]
Cobalamin <151 pmol/L	16 (26.7)	7 (22.6)	9 (31.0)	0.65
Ever Smoker	19 (31.7)	19 (61.3)	0 (0.0)	<0.01[g]
Ever Betel	16 (26.7)	7 (22.6)	9 (31.0)	0.65
Education >5 y	10 (16.7)	3 (9.7)	7 (24.1)	0.17[g]

Abbreviations used: bAs, blood arsenic; BMI, body mass index; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; PTHM, posttranslational histone modification; uAs, urinary arsenic; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine; uCr, urinary creatinine

[a]Values are median (range) or *n* (%) for continuous and categorical variables, respectively
[b]Wilcoxon rank-sum test or Chi-square test for difference between men and women in continuous and categorical variables, respectively
[c]*n* = 28 for women
[d]*n* = 58 for whole sample, *n* = 29 for men
[e]*n* = 57 for whole sample, *n* = 29 for men, *n* = 28 for women
[f]*n* = 60 for whole sample, *n* = 31 for men, *n* = 29 for women
[g]Fisher's exact test

Table S2. Baseline characteristics[a] of FACT participants with vs. without PTHM measures and with complete information for variables included in regression models

Characteristic	Participants with PTHM Measures (*n* = 317)	Participants without PTHM Measures (*n* = 293)	*P*[b]
Age (years)	39 (24-54)	37 (24-55)	0.03
BMI (kg/m^2)	19.3 (13.9-31.6)	19.6 (14.3-27.6)	0.27
uCr (μg/L)	48 (6-252)	41 (6-303)	<0.01
bAs (μg/L)	8.8 (1.0-80.2)	8.8 (1.8-35.0)	0.43
uAs (μg/L)	121 (11-1770)	109 (7-769)	0.05
uAs_{Cr} (μg/g uCr)	257 (35-2200)	275 (46-1100)	0.35
Men	161 (50.8)	146 (49.8)	0.81
Folate <9 nmol/L	74 (23.3)	46 (15.7)	0.02
Cobalamin <151 pmol/L	77 (24.3)	67 (22.9)	0.68
Ever Smoker	93 (29.3)	73 (24.9)	0.22
Ever Betel	87 (27.4)	63 (21.5)	0.09
Education >5 y	71 (22.4)	83 (28.3)	0.09

Abbreviations used: bAs, blood arsenic; BMI, body mass index; FACT, Folic Acid and Creatine Trial; PTHM, posttranslational histone modification; uAs, urinary arsenic; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine; uCr, urinary creatinine

[a]Values are median (range) or *n* (%) for continuous and categorical variables, respectively
[b]Wilcoxon rank-sum or Chi-square test for difference between those with vs. without PTHM measures for continuous and categorical variables, respectively

Table S3. Spearman correlation coefficients[a] for baseline measures of arsenic exposure and PTHMs in FACT participants

PTHM	Whole Sample		Men		Women	
	bAs	uAs_{Cr}	bAs	uAs_{Cr}	bAs	uAs_{Cr}
Unadjusted						
H3K36me2[b]	0.04 (0.44)	0.02 (0.68)	0.18 (0.02)	0.21 (<0.01)	-0.11 (0.18)	-0.13 (0.10)
H3K36me3[c]	-0.01 (0.85)	0.01 (0.81)	0.09 (0.26)	0.11 (0.18)	-0.10 (0.22)	-0.09 (0.28)
H3K79me2[d]	0.02 (0.68)	0.00 (0.97)	0.02 (0.78)	0.04 (0.61)	0.03 (0.74)	-0.03 (0.70)
Adjusted[e]						
H3K36me2[b]	0.04 (0.50)	0.03 (0.59)	0.17 (0.04)	0.20 (0.01)	-0.11 (0.17)	-0.15 (0.06)
H3K36me3[c]	0.00 (0.98)	0.01 (0.85)	0.08 (0.33)	0.11 (0.16)	-0.09 (0.26)	-0.09 (0.30)
H3K79me2[d]	0.01 (0.88)	-0.01 (0.85)	0.02 (0.76)	0.03 (0.69)	0.03 (0.74)	-0.02 (0.77)

Abbreviations used: bAs, blood arsenic; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; PTHM, posttranslational histone modification; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine

[a]Values presented are Spearman correlation coefficients, with respective *P*-values indicated in parentheses
[b]n = 311 for whole sample, n = 158 for men, n = 153 for women
[c]n = 300 for whole sample, n = 153 for men, n = 147 for women
[d]n = 315 for whole sample, n = 161 for men, n = 154 for women
[e]Partial Spearman correlation coefficients, adjusted for age, education, body mass index. Whole sample analyses were also adjusted for sex.

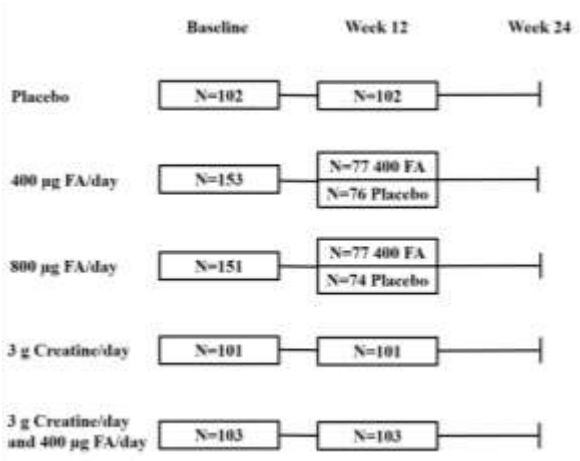
Table S4. Estimated regression coefficients[a] and 95% confidence intervals for associations between baseline measures of arsenic exposure and PTHMs, after adjusting for additional covariates, in FACT participants

PTHM	Arsenic Exposure	Whole Sample	Men	Women	*P*[b]
H3K36me2[c]	bAs	0.02 (-0.05, 0.09)	0.10 (-0.02, 0.22)	-0.04 (-0.12, 0.04)	0.06
	uAs_{Cr}	0.01 (-0.05, 0.08)	0.12 (+0.00, 0.23)*	-0.06 (-0.14, 0.02)	0.01
H3K36me3[d]	bAs	0.00 (-0.07. 0.06)	0.06 (-0.05, 0.16)	-0.05 (-0.15, 0.04)	0.12
	uAs_{Cr}	-0.01 (-0.08, 0.05)	0.07 (-0.03, 0.17)	-0.04 (-0.13, 0.05)	0.10
H3K79me2[e]	bAs	0.04 (0.05, 0.12)	0.06 (-0.07, 0.19)	0.03 (-0.08, 0.14)	0.75
	uAs_{Cr}	0.02 (-0.06, 0.10)	0.06 (-0.07, 0.19)	0.00 (-0.10, 0.11)	0.49

Abbreviations used: bAs, blood arsenic; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; PTHM, posttranslational histone modification; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine

[a]Estimated regression coefficients and 95% confidence intervals (β (CI)) from generalized linear models. Associations were examined between log(bAs) or log(uAs_{Cr}) in relation to each of the three PTHMs. Coefficients from H3K36me2 models indicate the change in the harmonic mean of H3K36me2 for a unit increase in the log-transformed arsenic measure, controlling for other covariates. Coefficients from H3K36me3 and H3K79me2 models indicate the change in the mean of the log(PTHM) for a unit increase in the log-transformed arsenic measure, controlling for other covariates. Models were adjusted for age, education (dichotomized at 5 years), log(BMI), log(uCr), log(plasma folate), log(plasma cobalamin), cigarette smoking status (ever vs. never), and betel nut chewing status (ever vs. never). Whole sample analyses were also adjusted for sex.
[b]Wald test for sex difference
[c]Whole sample n = 311, Males n = 158, Females n = 153.
[d]Whole sample n = 300, Males n = 153, Females n = 147.
[e]Whole sample n = 315, Males n = 161, Females n = 154.
*$P < 0.05$

A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Week 12
Week 24
Placebo
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N=102
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N=153
N=77 400 FA
N=76 Placebo
800 µg FA/day
N=151
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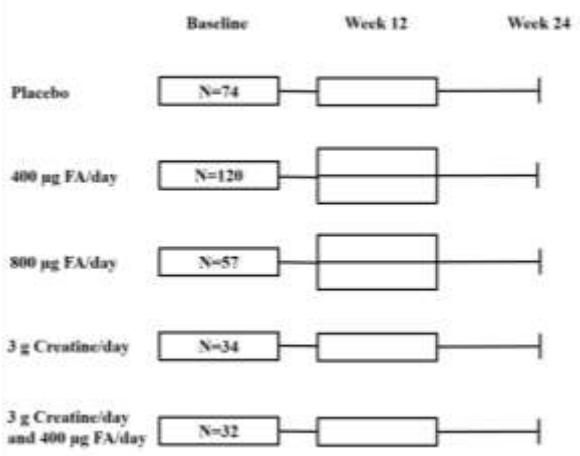
and 400 µg FA/day
N=103
N=103

B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Week 24
Placebo
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N=34
3 g Creatine/day
and 400 µg FA/day
N=32

C

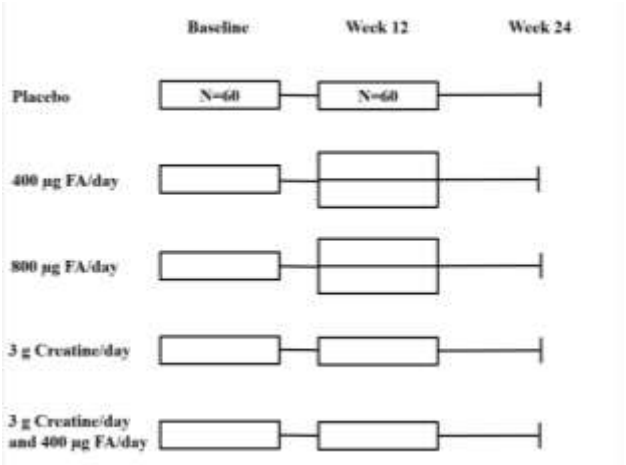

Baseline
Week 12
Week 24
Placebo
N=60
N=60
400 µg FA/day
800 µg FA/day
3 g Creatine/day
3 g Creatine/day
and 400 µg FA/day

Figure S1. Folic Acid and Creatine Trial (FACT) Study Design and Sampling for Current Study. **(A)** FACT study design. A total of 622 participants were randomized to five treatment arms. 12 participants were dropped during the course of the study. The final sample size for each treatment arm is shown. Approximately half of the participants in each folic acid (FA) treatment arm were switched to placebo at week 12. All participants received arsenic-removal water filters at baseline to be used for the duration of the 24 week study period and thereafter. **(B)** The first set of analyses for the current study used peripheral blood mononuclear cell (PBMC) samples collected at baseline from a total of 317 FACT participants. We show here the distribution of the 317 participants by treatment arm. By design, the majority of participants in the current study were from the placebo and the 400 µg FA/day treatment arms. To ensure sufficient statistical power for regression analyses, an additional 123 PBMC samples were selected from participants in other treatment arms who had measures for all covariates of interest. All cross-sectional analyses for the current study used PBMC samples that were collected from these 317 participants at baseline, prior to the provision of arsenic-removal water filters and nutritional supplements or a placebo **(C)** The second set of analyses for the current study used all available samples from the placebo group (n = 60). These samples were collected at baseline and at week 12. Participants in the placebo group did not receive any nutritional interventions during the study period but, like all other FACT participants, received arsenic-removal water filters at baseline to be used during the study period and thereafter. Abbreviations used: FA, folic acid; FACT, Folic Acid and Creatine Trial; PBMC, peripheral blood mononuclear cell

CHAPTER SIX

Sex-specific associations between arsenic exposure and DNA methylation and mRNA expression of candidate genes in Bangladeshi adults with arsenicosis

Caitlin G. Howe[1]*, Maria Argos[2]*, Farzana Jasmine[2], Faruque Parvez[1], Mahfuzar Rahman[3], Muhammad Rakibuz-Zaman[3], Vesna Slavkovich[1], John A. Baron[4], Joseph H. Graziano[1], Muhammad G. Kibriya[2], Mary V. Gamble[1], Habibul Ahsan[2, 5, 6]

*These authors contributed equally to this manuscript

Affiliations: [1]Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, New York, USA; [2]Department of Public Health Sciences, The University of Chicago, Chicago, Illinois, USA; [3]U-Chicago Research Bangladesh, Dhaka, Bangladesh; [4]Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina, USA; [5]Department of Medicine, and [6]Department of Human Genetics and Comprehensive Cancer Center, The University of Chicago, Chicago, Illinois, USA

ABSTRACT

Arsenic is a human carcinogen which has also been associated with numerous non-cancer health outcomes. Based on our prior research, we were interested in genes involved in pathways implicated in arsenic toxicity, including arsenic metabolism, one-carbon metabolism, epigenetic regulation, DNA repair, and tumor suppression; thus we selected 47 relevant candidate genes. For many arsenic-related health outcomes, susceptibility differs by sex. There is also increasing evidence that arsenic induces epigenetic dysregulation in a sex-dependent manner. Whether or not arsenic also alters gene expression in a sex-dependent manner has not been confirmed in a large epidemiological study. We therefore examined sex-specific associations between baseline measures of creatinine-adjusted urinary arsenic (uAs_{Cr}) and gene-specific DNA methylation (whole blood, n = 400) and mRNA expression (peripheral blood mononuclear cells, n = 1799) for the 47 selected candidate genes among participants in the Bangladesh Vitamin E and Selenium Trial. DNA methylation and mRNA expression levels were measured using Illumina's HumanMethylation450 BeadChip and HumanHT-12-v4 BeadChip kits, respectively. In linear regression analyses, which were adjusted for multiple comparisons using the Bonferroni correction, we observed that uAs_{Cr} was associated with methylation at five CpG sites within four of the candidate genes. Three of these CpG sites were differentially methylated by arsenic exposure among women only. In similar analyses, we observed that uAs_{Cr} was significantly associated with the expression of 18 candidate genes. Of these genes, six were differentially expressed by arsenic exposure among men only and five among women only. Our findings contribute to growing evidence that arsenic alters epigenetic modifications, such as DNA methylation, in a sex-dependent manner, and suggest that this may have functional consequences, such as alterations in gene expression.

INTRODUCTION

Exposure to arsenic-contaminated drinking water is a global problem. In Bangladesh alone, more than 57 million individuals are exposed to arsenic concentrations exceeding the World Health Organization guideline of 10 μg/L [1]. Exposure to inorganic arsenic has been associated with both cancer and non-cancer outcomes, with susceptibility often differing by sex [2]. For example, men are more prone to developing arsenic-induced skin lesions and skin, liver, and bladder cancers, while women and girls may be more susceptible to arsenic-induced cardiovascular disease and developmental outcomes, respectively ([3-9] and reviewed in [2]). Although gender-based differences in co-exposures may explain some of the differences in susceptibility, animal studies have similarly demonstrated sex-dependent effects of arsenic [10, 11], suggesting contributions from biological sex. While the mechanisms underlying these sex differences are likely multifactorial, there is evidence that arsenic induces epigenetic dysregulation, including alterations in DNA methylation and posttranslational histone modifications (PTHMs), and these effects may differ by sex [12-18]. Exposure to arsenic has also been shown to alter gene expression [19-22]. However, while one small human study (n = 29) has observed dramatic sex differences in the effects of arsenic on gene expression [23], this has not been confirmed in a larger epidemiological study.

The effects of arsenic on genome-wide DNA methylation and gene expression were previously investigated in participants from the Bangladesh Vitamin E and Selenium Trial (BEST) [24]. The original report evaluated all 485,577 CpG sites, representing >99% of RefSeq genes, on Illumina's HumanMethylation450 platform, and differences between men and women were not examined. For the current study, we used a candidate gene approach and evaluated the relationships between arsenic exposure, gene-specific DNA methylation, and mRNA expression

in BEST study participants separately by sex. For our candidate genes, we selected a total of 47 genes which are involved in arsenic metabolism, one-carbon metabolism (OCM), epigenetic regulation, DNA repair, and tumor suppression, since these pathways have been implicated in arsenic metabolism and toxicity [25-30]. Our approach for identifying candidate genes was two-pronged: 1) we used the Comparative Toxicogenomics Database (CTD) [31] to identify genes involved in our pathways of interest that have previously been shown to be affected by arsenic exposure (32 genes) and 2) we selected additional genes in these same pathways that were not necessarily identified by the CTD as known targets of arsenic (15 genes).

STUDY PARTICIPANTS AND METHODS

Study Population

For the current study, we used baseline-collected (i.e., pre-intervention) samples from 1799 participants in the BEST study [32]. These participants were the first 1799 individuals enrolled into the study. Of these 1799 participants, 400 were randomly selected for DNA methylation measures for an epigenome-wide study, which has been published [24]. The BEST study is a 2x2 factorial randomized chemoprevention trial, which was designed to examine whether vitamin E and/or selenium supplementation prevent non-melanoma skin cancer risk in Bangladeshi adults with arsenicosis. Participants were eligible for the BEST study if they were between the ages of 25 and 65, had a permanent residence within the study area in central Bangladesh, exhibited manifest arsenical skin lesions, and had no prior history of cancer. Individuals who met these eligibility criteria (n = 7000) were enrolled between April 2006 and August 2009.

Urinary Arsenic

Total urinary arsenic was measured in baseline-collected urine samples by graphite furnace atomic absorption spectrometry (AAnalyst 600 spectrometer; PerkinElmer, Norwalk, CT, USA) in the Trace Metals Core Lab at Columbia University [33]. The intra- and inter-assay CVs were 6.2% and 4.4%, respectively. Total urinary arsenic concentrations were adjusted for urinary creatinine, which was measured by a method based on the Jaffe method [34]. The intra- and inter-assay CVs for creatinine were 3.5% and 2.2%, respectively.

Selection of Candidate Genes

The 47 candidate genes selected for the current study are listed in **Table 1**. The CTD was used to identify genes in our pathways of interest that had previously been examined in relation to arsenic exposure. To identify all genes affected by arsenic exposure at the gene, mRNA, and protein level, we entered "arsenic" as our chemical of interest in the CTD Chemical-Gene Interaction Query. Of the genes identified as targets of arsenic, we then searched for those involved in the one-carbon metabolism pathway, arsenic metabolism, epigenetic regulation (with a particular focus on DNA methyltransferases and histone modifying enzymes), DNA repair, and tumor suppression/oncogenesis. The CTD is updated every month; we used the August 2015 release.

DNA Methylation

DNeasy Blood kits (Qiagen, Valencia, CA, USA) were used to isolate DNA from whole blood. DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA), and 500 ng was used for measuring DNA methylation by Illumina's HumanMethylation450 BeadChip kit (Illumina, San Diego, CA, USA). Quantile normalization

was applied to the methylation score (β), which is a continuous measure from 0 to 1 for each CpG site, with 0 representing no methylation and 1 representing complete methylation. Of the 413 samples for which DNA methylation was measured, 13 were excluded for reasons that have been described previously [24]. β values with a *P* for detection > 0.05 were excluded. A logit-transformation was applied to quantile-normalized β values, and ComBat software was used to adjust for potential batch effects [35].

Gene Expression

As described previously [24], RNA was isolated from peripheral blood mononuclear cells, which had been stored at -80ºC in Buffer RLT, using the RNeasy Micro Kit from QIAGEN (Valencia, CA, USA). Illumina's TotalPrep 96 RNA Amplification kit was used to synthesize cRNA from 250 ng RNA, and 750 ng of cRNA was used to measure gene expression via Illumina's HumanHT-12-v4 BeadChip kit, which evaluates >30,000 genes. Gene expression data were available for 1799 participants, including the 400 participants for which there was also complete DNA methylation data. A $\log_2$-transformation was applied to quantile normalized mRNA expression values, and ComBat software was used to adjust for potential batch effects [35].

Statistical Methods

Separate linear regression models were used to examine associations between creatinine-adjusted urinary arsenic (uAs_{Cr}), measured continuously, and the logit-transformed β for each CpG site or the $\log_2$-transformed expression value for each gene. A total of 1959 CpG sites and 78 mRNA transcripts, representing the 47 candidate genes, were evaluated. Models were run in all participants, and were adjusted for age and sex. Analyses were also run separately by sex and

adjusted for age. Since arsenic was not associated with white blood cell type fractions [24], which were estimated using the method by Houseman et al. [36], we did not adjust for cell type distribution in our models. A Bonferroni correction was used to adjust for multiple comparisons, which was based on the number of probes representing each gene.

RESULTS

General characteristics of the study participants have been reported previously [24]. Participants were between 25 and 65 years old. 53% of the participants were male. The mean (± SD) uAs_{Cr} concentration was 302 ± 365 µg/g creatinine for the 1799 participants with gene expression measures and 302 ± 365 µg/g creatinine for the subset that also had DNA methylation measures.

DNA Methylation

Before correcting for multiple comparisons, a total of 178 CpG sites (out of 1959 CpG sites), representing 40 of the 47 candidate genes, were differentially methylated by arsenic exposure ($P < 0.05$), either in the whole sample or in men or women alone (**Supplemental Material, Table S1**). When restricting to sex-stratified analyses, a total of 146 CpG sites were differentially methylated by arsenic exposure; 51 sites were differentially methylated in men only, 94 in women only, and 1 site (cg03315649), located in a CpG island within the gene body of *KDM4B*, was differentially methylated in both men (t-statistic: -2.14, $P = 0.033$) and women (t-statistic: -3.01, $P = 0.003$).

Table 1. Candidate genes

Pathway	Gene	Target of arsenic according to CTD?	Additional reasons for inclusion
Arsenic metabolism			
	AS3MT	Unknown	Evidence that arsenic inhibits its own methylation (PMIDs: 10409394, 24598884) Evidence that arsenic induces *AS3MT* expression in human PBMCs *in vitro* (PMID: 24154821)
One-carbon metabolism			
	AHCY*	Yes	
	BHMT	Yes	
	CBS*	Yes	
	DHFR*	Yes	
	GAMT*	Unknown	Arsenic reduces hepatic creatine concentrations in mice (PMID: 25753946)
	GNMT*	Unknown	
	GSS*	Yes	
	MAT1A	Yes	
	MAT2A	Yes	
	MAT2B	Yes	
	MTHFD1	Unknown	Arsenic alters MTHFD1 protein levels post-translationally (Patrick Stover, Cornell University, personal communication), but potential effects on DNA methylation and mRNA expression unknown
	MTHFR	Unknown	
	MTR*	Unknown	
	MTRR	Unknown	
	PEMT	Unknown	Evidence that choline is altered by arsenic in rodents (PMID: 24448970, PMID: 19073202, 23085348) and potentially humans (PMID: 27052531)

Epigenetic regulation			
	DNMT1	Yes	
	DNMT3A	Yes	
	DNMT3B	Yes	
	DOT1L	Unknown	Related to 5-hmC, which is altered by arsenic in multiple tissues in rats (PMID: 25256144) and is associated with 5-hmC in human WBCs (PMID: 26364164)
	EHMT1	Yes	
	EHMT2	Yes	
	EZH2*	Yes	
	HDAC4*	Yes	
	HDAC5	Yes	
	KDM2B	Yes	Arsenic is positively associated with DNA methylation in the promoter region among men only (unpublished data). Arsenic is positively associated with its target, H3K36me2, in human PBMCs among men only (PMID: 26967670)
	KDM4B	Yes	
	KDM5B	Unknown	Altered by arsenic in mouse brain (PMID: 26193056)
	MLL*	Unknown	Altered by arsenic in mouse brain (PMID: 26193056). Arsenic exposure is also associated with methylation of its target, H3K4, in human PBMCs (PMID: 23064002) and WBCs (PMID: 21385672)
	NSD1	Unknown	Arsenic alters methylation of its target, H3K36, in cultured A549 cells (PMID: 18321869) and is associated with H3K36me2 in human PBMCs among men only (PMID: 26967670)
	PRDM2*	Yes	
	TET1	Unknown	Arsenic alters its product (5-hmC) in multiple rat tissues (PMID: 25256144) and is associated with 5-hmC in human WBCs in a sex-dependent manner (PMID: 26364164)
	TET2*	Yes	Arsenic alters its product (5-hmC) in multiple rat tissues (PMID: 25256144) and is associated with 5-hmC in human WBCs in a sex-dependent manner (PMID: 26364164)
	TET3	Unknown	Arsenic alters its product (5-hmC) in multiple rat tissues (PMID: 25256144) and is associated with 5-hmC in human WBCs in a sex-dependent manner (PMID: 26364164)

DNA repair			
	MSH2*	Yes	
	OGG	Yes	
	PARP1	Yes	
	PCNA	Yes	
	POLB*	Yes	
	XRCC1	Yes	
	XRCC2	Unknown	
	XRCC3*	Yes	
	XRCC4	Yes	
	XRCC5*	Yes	
	XRCC6*	Unknown	
Tumor suppression			
	EGFR	Yes	
	P53*	Yes	

Abbreviations used: 5-hmC, 5-hydroxymethylcytosine; CTD, Comparative Toxicogenomics Database; H3K4, lysine 4 of histone H3; H3K36, lysine 36 of histone H3; H3K36me2, di-methylation at lysine 36 of histone H3; PBMC, peripheral blood mononuclear cell; WBC, white blood cell

*In the current study, mRNA expression was found to be significantly associated with arsenic exposure in either men, women, or the whole sample, after adjusting for multiple comparisons

After adjusting for multiple comparisons using a Bonferroni correction (based on the number of probes per gene), arsenic exposure was associated with differential methylation of 5 CpG sites located in 4 genes, either in the whole sample, or in men or women alone (**Table 2, Figure 1**). Differences by sex were observed for 3 of the 4 differentially methylated genes. Among women, but not men, arsenic exposure was inversely associated with methylation at a CpG site (cg02978542) within the transcription start site (TSS) of *MTHFR*. In contrast, among women, but not men, arsenic exposure was positively associated with methylation at a CpG site (cg05998850) located within the gene body of *XRCC5*. Among both men and women, arsenic exposure was negatively associated with methylation at a CpG site (cg03315649) located within the gene body of *KDM4B*. Arsenic exposure was also inversely associated with methylation levels at two CpG sites within *PCNA*. However, one site (cg09011324), located in the TSS of *PCNA*, was only differentially methylated among women, whereas the other site (cg22960971), located within the gene body, was differentially methylated among both men and women (Table 1). Arsenic exposure was also positively associated with the methylation of two CpG sites within the gene body of *AS3MT*, although this was not statistically significant after adjusting for multiple comparisons (Supplemental Material, Table S1).

mRNA Expression

Before correcting for multiple comparisons, 22 of the 47 genes examined were found to be differentially expressed by arsenic exposure ($P < 0.05$), either in the whole sample or in men or women alone (**Supplemental Material, Table S2**). When restricting to sex-stratified analyses, 14 genes were significantly associated with arsenic exposure among men and 11 genes were significantly associated with arsenic exposure among women. Of these genes, only 6 were

significantly associated with arsenic exposure in both men and women: *GSS*, *EZH2*, *MLL*, *POLB*, *PRDM2*, and *XRCC6*.

After adjusting for multiple comparisons, arsenic exposure was associated with differential expression of 18 genes (**Table 3,** Figure 1). Of these genes, 6 were differentially expressed among men only, and 5 among women only. There was also a suggestive inverse association between arsenic exposure and *AS3MT* expression among men ($P = 0.051$) (data not shown).

Genes that were both differentially methylated and expressed by arsenic exposure

After correcting for multiple comparisons, *XRCC5* was the only gene for which both DNA methylation and gene expression were significantly associated with arsenic exposure. Among women, arsenic exposure was positively associated with methylation at a CpG site (cg05998850) within the gene body of *XRCC5* ($P = 0.002$) and was also negatively associated with *XRCC5* mRNA levels ($P = 0.003$) (Tables 1 and 2, Figure 1).

Table 2. Genes that were differentially methylated by arsenic exposure after adjusting for multiple comparisons, either in the whole sample, in men, or in women

	CpG Label	CpG Location	# Probes	*P* Cutoff[a]	Whole Sample (*n* = 400)		Men (*n* = 212)		Women (*n* = 188)	
					t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
One-Carbon Metabolism										
MTHFR	cg02978542	1st Exon/TSS200/ 5'UTR, Island	24	0.002	-3.08	0.002	-0.76	0.446	-3.48	6.30 E-04*
Epigenetic Regulation										
KDM4B	cg03315649	Body, Island	118	4.24 E-4	-3.67	2.80 E-04*	-2.14	0.033	-3.01	0.003
DNA Repair										
PCNA	cg09011324	TSS200/5'UTR, Island	15	0.003	-2.64	0.009	-0.14	0.890	-3.66	3.30 E-04*
PCNA	cg22960971	Body/N_Shore	15	0.003	-3.49	5.30 E-04*	-1.92	0.056	-2.98	0.003*
XRCC5	cg05998850	Body	20	0.003	1.12	0.265	-1.75	0.082	3.14	0.002*

Abbreviations used: CpG, cytosine guanine dinucleotide; TSS200, within 200 basepairs of a transcription start site; 5'UTR, within the 5' untranslated region; N_Shore, within North shore

[a]*P* cutoff is based on an alpha of 0.05, which was adjusted using the Bonferroni method to account for the number of probes representing the gene on the microarray

[b]t-statistic and corresponding *P*-value are from linear regression models. Whole sample analyses were adjusted for age and sex. Sex-stratified analyses were adjusted for age.

**P* value was statistically significant after adjusting for multiple comparisons, using the Bonferroni correction

Table 3. Genes that were differentially expressed by arsenic exposure after adjusting for multiple comparisons, either in the whole sample, in men, or in women

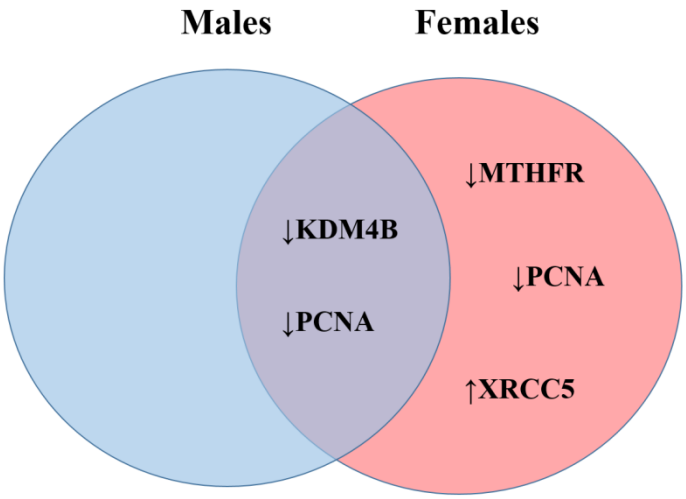
	Probe Label	# Probes	*P* Cutoff[a]	Whole Sample (*n* = 1799)		Men (*n* = 991)		Women (*n* = 808)	
				β[b]	*P*	β[b]	*P*	β[b]	*P*
One-carbon metabolism									
AHCY	ILMN_1657862	1	0.050	2.62 E-05	0.110	4.27 E-05	0.036*	2.14 E-06	0.936
CBS	ILMN_1804735	1	0.050	2.28 E-05	0.035*	1.06 E-05	0.448	4.05 E-05	0.019*
DHFR (variant 1)	ILMN_1759872	2	0.025	6.59 E-05	0.008*	7.01 E-05	0.022*	6.19 E-05	0.133
GAMT (variant 1)	ILMN_1756469	3	0.017	-3.98 E-05	0.001*	-2.37 E-05	0.133	-6.49 E-05	0.001*
GAMT (variant 1)	ILMN_1794595	3	0.017	-1.89 E-05	0.091	-9.50 E-07	0.947	-4.72 E-05	0.008*
GNMT	ILMN_1736238	1	0.050	3.62 E-06	0.578	-7.62 E-06	0.350	2.12 E-05	0.044*
GSS	ILMN_1683462	1	0.050	3.66 E-05	1.08 E-04*	4.04 E-05	8.64 E-04*	3.22 E-05	0.033*
MTR	ILMN_1670801	1	0.050	-2.81 E-05	0.022*	-3.24 E-05	0.043*	-2.17 E-05	0.262
Epigenetic regulation									
EZH2 (variant 2)	ILMN_1708105	3	0.017	4.09 E-05	1.46 E-05*	3.29 E-05	0.007*	5.18 E-05	5.28 E-04*
EZH2 (variant 2)	ILMN_2364529	3	0.017	3.58 E-05	1.30 E-04*	2.86 E-05	0.015*	4.53 E-05	0.003*
EZH2 (variant 1)	ILMN_1652913	3	0.017	3.17 E-05	0.003*	2.72 E-05	0.047	3.83 E-05	0.028
HDAC4	ILMN_1764396	1	0.050	-2.32 E-05	0.071	-3.78 E-05	0.017*	-8.31 E-07	0.969
MLL	ILMN_1668683	1	0.050	-6.59 E-05	6.80 E-08*	-5.45 E-05	4.92 E-04*	-8.13 E-05	2.86 E-05*
PRDM2 (variant 2)	ILMN_1652992	4	0.013	-2.03 E-05	0.002*	-1.98 E-05	0.026	-2.07 E-05	0.038
TET2	ILMN_1788818	1	0.050	2.18 E-05	0.008*	-3.19 E-06	0.757	5.94 E-05	8.23 E-06*
Tumor suppression									
TP53	ILMN_1779356	1	0.050	2.47E-05	0.075	4.93E-05	0.006*	-9.68E-06	0.660
DNA repair									
MSH2	ILMN_1737413	2	0.025	-2.42E-05	0.016*	-2.05E-05	0.116	-3.08E-05	0.051
POLB	ILMN_1767894	1	0.050	5.01E-05	3.77 E-05*	3.85E-05	0.016*	6.99E-05	2.15 E-04*
XRCC3	ILMN_1696266	1	0.050	9.88 E-06	0.420	3.13 E-05	0.047*	-2.18 E-05	0.262
XRCC5	ILMN_2105983	1	0.050	-2.84E-05	0.015*	-9.92E-06	0.509	-5.47E-05	0.003*
XRCC6	ILMN_1743097	2	0.025	-4.61E-05	1.96 E-04*	-3.60E-05	0.023*	-6.24E-05	0.002*

[a]*P* cutoff is based on an alpha of 0.05, which was adjusted using the Bonferroni method to account for the number of probes representing the gene on the microarray

[b]β and corresponding *P*-value are from linear regression models. Whole sample analyses were adjusted for age and sex. Sex-stratified analyses were adjusted for age.

**P* value was statistically significant after adjusting for multiple comparisons, using the Bonferroni correction

A



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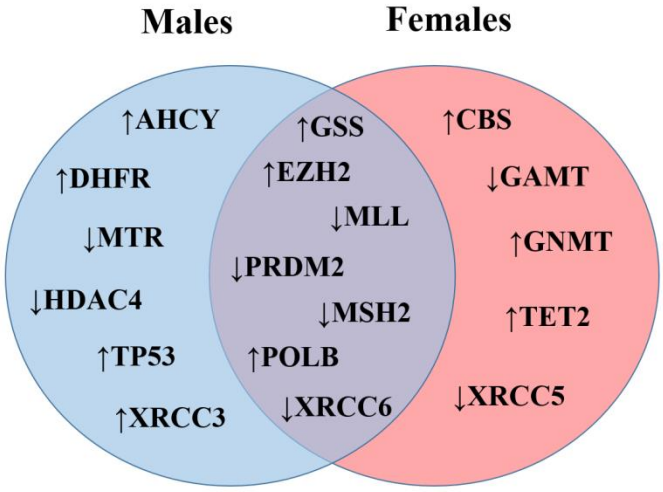

Figure 1. Venn diagrams representing genes that were differentially methylated (**A**) or expressed (**B**) by arsenic exposure in males (blue) compared with females (pink). The intersections show genes that were differentially methylated or expressed in both males and females. Arrows indicate whether associations with urinary arsenic exposure were positive (↑) or negative (↓). Sample sizes for DNA methylation: 212 for men, 188 for women. Sample sizes for mRNA expression: 991 for men, 808 for women

Comparison with previous findings

Many, but not all, of our findings were consistent with previous studies (**Tables 4** and **5**). However, we also observed several novel associations between arsenic exposure and the methylation or expression of genes involved in the OCM pathway. For example, we observed a significant inverse association between arsenic exposure and the methylation of *MTHFR*, which was driven by women. Among women, arsenic exposure was also positively associated with the expression of *GNMT* and negatively associated with the expression of *GAMT*.

Table 4. Comparison of DNA methylation findings with previous studies

Differentially methylated genes	**Our findings**	**Findings from previous studies**
One-carbon metabolism		
MTHFR	Negatively associated with cg02978542 methylation in females	No previous studies
Epigenetic Regulation		
KDM4B	Negatively associated with cg03315649 methylation in both males and females	PMID: 25304211: Prenatal arsenic exposure positively associated with methylation of a different CpG site (cg13559217) in cord blood (males and females combined)
DNA Repair		
PCNA	Negative association with cg09011324 methylation in females Negative association with cg22960971 methylation in both males and females	PMID: 12899209: Higher protein expression in skin tissue from individuals with worsening degrees of arsenicosis. (Proportion of males and females not specified). PMID: 15345372: Increased mRNA in livers of arsenic-exposed female Tg.AC mice PMID: 16507464: Increased mRNA in livers of adult male C3H mice exposed to arsenic *in utero* PMID: 16876216: Arsenic-induced increase in mRNA in arsenic-transformed rat liver epithelial cells (TRL1215) (sex unknown)
XRCC5	Positive association with cg05998850 methylation in females	PMID: 19818359: Increased protein expression in arsenic-exposed E7 immortalized human uroepithelial cells (sex unknown)

Abbreviations used: CpG, Cytosine-guanine dinucleotide

Table 5. Comparison of mRNA expression findings with previous studies

Differentially expressed genes	Our findings	Findings from previous studies
One-Carbon Metabolism		
AHCY	Positive association in males	PMID: 18487201: Increased mRNA and protein expression in arsenic-exposed human prostate epithelial cell line
CBS	Positive association, only significant in females	PMID: 25304211: Prenatal arsenic (maternal arsenic) exposure positively associated with cg11815682 methylation in newborn cord blood (males and females combined) PMID: 18487201: Increased mRNA expression in arsenic-exposed human prostate epithelial cell line
DHFR	Positive association, only significant in males	PMID: 25304211: Prenatal arsenic (maternal arsenic) exposure negatively associated with cg08244028 methylation in newborn cord blood (males and females combined)
GAMT	Negative association in females	No previous studies
GNMT	Positive association in females	No previous studies
GSS	Positive association in both males and females	PMID: 18487201: Protein expression increased in arsenic-exposed prostate epithelial cell line PMID: 12634122: mRNA expression decreased in arsenic-exposed human keratinocytes (male-derived)
MTR	Negative association, only significant in males	PMID: 25697676: Increased mRNA in serum from arsenic-exposed male rats
Epigenetic Regulation		
EZH2	Positive association in both males and females (Variant 2, ILMN_1708105). Positive association in both males and females (Variant 2, ILMN_2364529) Positive association in both males and females (Variant 1, ILMN_1652913)	PMID: 22426358: Increased protein expression in arsenic-exposed C2C12 cells (immortalized mouse myoblast cells, female-derived) PMID: 22843710: Increased protein expression in BALB/c 3T3 cells (mouse embryonic fibroblast cells, female-derived)
HDAC4	Negative association, only significant in males	PMID: 25304211: Prenatal arsenic exposure positively associated with methylation at five CpG sites (cg16202803, cg06107260, cg16360836, cg26673264, cg11707035) and negatively associated with mRNA expression in newborn cord blood (males and females combined)
MLL	Negative association in both males and females	PMID: 26193056: Increased protein expression in male mouse dentate gyrus, decreased in female mouse dentate gyrus
PRDM2	Negative association in both males and females (Variant 2, ILMN_1652992)	PMID: 21291286: Higher methylation in promoter region in peripheral blood from individuals with arsenic-induced skin lesions (males and females combined) PMID: 26039340: Hypomethylation at promoter region in exfoliated bladder cells from arsenic-exposed adults (males and females combined) PMID: 12776498: Decreased mRNA in NB4 cells (female-derived)
TET2	Positive association, driven by females	PMID: 25304211: Prenatal arsenic exposure positively associated with cg09295382 methylation in newborn cord

		blood (males and females combined)
Tumor Suppression		
TP53	Positive association in males only	PMID: 12899209: Higher protein expression in skin samples from individuals with worsening degrees of arsenicosis (proportion of males and females not specified) PMID: 19945496: Decreased expression in BEAS-2B cells (human bronchial epithelial cell line, male-derived) PMID: 21756780: Increased methylation in blood from those with more severe arsenicosis (sex of participants unspecified) PMID: 23174854: Increased expression in arsenic-exposed keratinocytes (HaCaT, male-derived) PMID: 16251483: Increased methylation in blood from those exposed to arsenic and in those with arsenic-induced skin cancer vs. skin cancer not caused by arsenic exposure (males and females combined) PMID: 11813266: Increased protein expression in human lymphoblastoid cell lines (male-derived) PMID: 11507245: Increased protein expression in human lymphoblastoid cell lines derived from patients with ataxia telangiectasia (sex unspecified)
DNA Repair		
MSH2	Negative association in both males and females	PMID: 12634122: Decreased mRNA in human keratinocytes (male-derived) PMID: 17450239: Non-significant decrease in mRNA in skin tissue from those with high arsenic exposure compared with controls (proportion of males and females not specified)
POLB	Positive association in both males and females	Snow et al.[a]: Increased protein expression in HaCaT cells (male-derived) and GM847 cells (immortalized human fibroblasts, sex unknown) PMID: 21776218: Increased mRNA in AC16 cells (cardiomyocytes, female-derived) PMID: 21332098: Negative association with mRNA and protein expression in blood from healthy individuals (males and females combined, proportions not specified)
XRCC3	Positive association in males	PMID: 17530438: Increased in arsenic-exposed U87MG cells (human glioma cell line, male-derived)
XRCC5	Negative association, driven by females	PMID: 19818359: Increased protein expression in bladder epithelial cells (sex unspecified)
XRCC6	Negative association in males and females (ILMN_1743097)	PMID: 16014739: Decreased expression in mouse embryonic fibroblasts (male- and female-derived) PMID: 12016162: Increased expression in human fibroblasts (male-derived)

[a]Snow ET, Schuliga M, Chouchane S, Hu Y (2001). Sub-toxic arsenite induces a multi-component protective response against oxidative stress in human cells in Arsenic Exposure and Health Effects IV. Chappell WR, Abernathy CO, Calderon RL (Eds.). New York, New York: Elsevier Science Ltd.

DISCUSSION

We observed that arsenic exposure was associated with the methylation and/or expression of several candidate genes involved in OCM, DNA repair, epigenetic regulation, and tumor suppression/oncogenesis. These associations often differed between men and women, consistent with previous reports which have shown that arsenic alters epigenetic modifications, including DNA methylation and PTHMs, in a sex-dependent manner [12-18].

Among women, arsenic exposure was negatively associated with methylation at a CpG site (cg02978542) within the TSS of *MTHFR*, an enzyme involved in folate metabolism. *MTHFR* methylation and expression have not previously been examined in relation to arsenic exposure. Given the findings for *MTHFR* methylation, we would have expected arsenic exposure to be associated with increased *MTHFR* expression, which would likely be protective, as folate is a methyl donor which facilitates arsenic metabolism and excretion [37, 38], and folate deficiency is a risk factor for arsenic-induced skin lesions [39]. However, we did not observe significant associations between arsenic exposure and *MTHFR* expression in this study.

After correcting for multiple comparisons, *KDM4B* was differentially methylated by arsenic exposure in the whole study sample, and in the same direction for both men and women. *KDM4B* codes for a histone demethylase, which removes a single methyl group from the tri-methylated forms of lysines 9 and 36 on histone H3 (H3K9me3 and H3K36me3, respectively) [40]; this leads to increased levels of their di-methyl forms (H3K9me2 and H3K36me2, respectively). We have previously observed that arsenic exposure is associated with higher global levels of H3K9me2 among both men and women [13] and with higher global levels of H3K36me2 among men [18]. Thus, arsenic-induced alterations in histone demethylases, such as *KDM4B*, may explain some of the observed effects of arsenic on PTHMs.

After correcting for multiple comparisons, *XRCC5* was the only gene that was both differentially methylated and differentially expressed by arsenic exposure. *XRCC5* assists with non-homologous end joining (NHEJ), a mechanism that is important for both DNA repair and V(D)J recombination [41]. Among women, arsenic was positively associated with methylation at a CpG site (cg05998850) within the gene body of *XRCC5* and was negatively associated with *XRCC5* mRNA expression. Recent evidence suggests that methylation at certain intragenic CpG sites is more highly correlated with mRNA levels than is methylation within promoter regions [42]. Therefore, it is possible that methylation at cg05998850 regulates *XRCC5* expression, which should be explored in future studies. In contrast with our findings, a previous *in vitro* study by Chen et al. observed higher *XRCC5* protein expression in arsenic-exposed bladder epithelial cells [43]. It is possible that tissue and/or sex differences may have contributed to this discrepancy, though it is not clear from what sex the cell line used by Chen et al. was derived.

Several other genes involved in DNA repair were differentially expressed by arsenic exposure. Consistent with previous *in vitro* studies [44-47], arsenic exposure was inversely associated with expression levels of *XRCC5*, *XRCC6*, and *MSH2*, which are enzymes involved in NHEJ and mismatch repair, respectively [41, 48]. Inhibition of DNA repair is one hypothesized mechanism of arsenic carcinogenicity; in particular, arsenic has been shown to inhibit the activity of DNA repair enzymes which contain zinc finger domains, such as PARP-1 [49]. Our study, along with several others [44-47], suggests that arsenic may also reduce the expression of key enzymes involved in DNA repair pathways, including NHEJ and mismatch repair. However, three of the candidate genes involved in DNA repair or damage responses did not follow this trend. Consistent with previous studies [50-52], we observed that arsenic exposure was positively associated with the expression of *POLB*, a DNA polymerase involved in base excision repair

[53], and also with *XRCC3*, which is involved in homologous recombination [41], although only among men. Furthermore, we did not observe associations between arsenic exposure and the expression of *PCNA*, a DNA clamp which is involved in both normal DNA replication and the DNA damage response [54].

Although the role of OCM in facilitating arsenic metabolism has been well-studied [25], the effects of arsenic on the OCM pathway are less well understood. However, two metabolomics studies in mice have observed that several metabolites involved in the OCM pathway, including betaine, choline, homocysteine, and methionine, are altered by arsenic [55, 56]. Furthermore, a previous *in vitro* study, which utilized a normal human prostate epithelial cell line, observed that arsenic altered the expression of several genes involved in the OCM pathway [57]. In particular, they found that genes involved in the synthesis of glutathione, the primary endogenous antioxidant, were upregulated in response to arsenic exposure [57]. Similarly, we observed that arsenic exposure was positively associated with the expression of two genes involved in glutathione synthesis: *CBS* among women and *GSS* among both men and women. Several other genes in the OCM pathway were also differentially expressed by arsenic exposure, including *AHCY*, *DHFR*, *GAMT*, *GNMT*, and *MTR*, and many of these associations differed by sex (**Figure 2**). The effects of arsenic on *GAMT*, an enzyme involved in creatine synthesis [58], had not been examined previously. However, arsenic has been shown to reduce hepatic mitochondrial creatine concentrations in mice [56]. Consistent with this, we observed that arsenic exposure was negatively associated with *GAMT* expression, but only among women. We also observed a positive association between arsenic exposure and *GNMT* expression among women, but not men. *GNMT* codes for an enzyme which regulates concentrations of *S*-adenosylmethionine (SAM), the universal methyl donor [59]. Arsenic reduces SAM

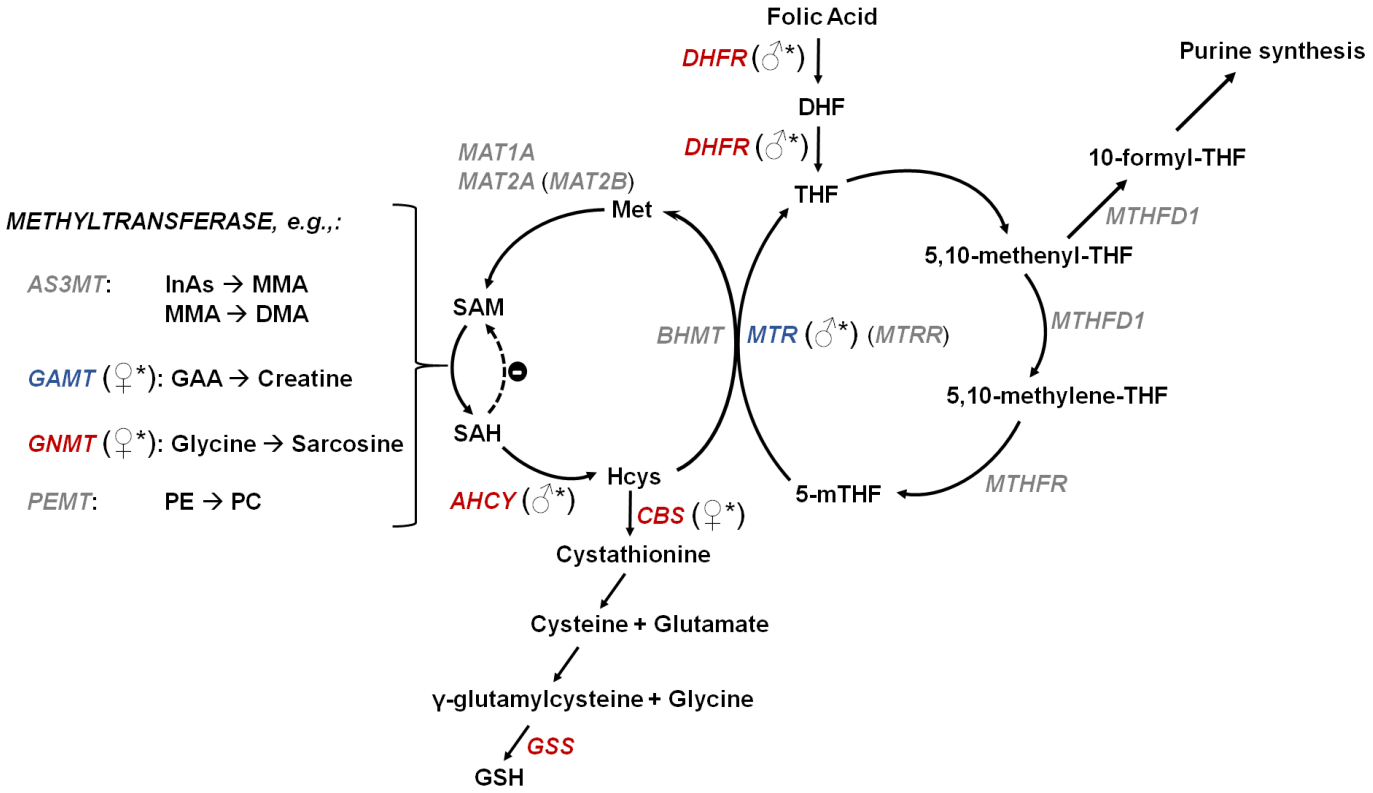

Figure 2. One-carbon metabolism genes that were differentially expressed by arsenic exposure, after adjusting for multiple comparisons. Candidate genes are italicized. Positive associations between arsenic exposure and mRNA expression were observed for genes indicated in red. Negative associations between arsenic exposure and mRNA expression were observed for genes indicated in blue. Significant associations were not observed between arsenic exposure and mRNA expression for genes indicated in gray. (♂*): statistically significant among men only. (♀*): statistically significant among women only. Abbreviations used: 5-mTHF, 5-methyl-tetrahydrofolate; 5,10-mTHF, 5-methylene-tetrahydrofolate; AHCY, *S*-adenosylhomocysteine hydrolase; AS3MT, arsenic (+3 oxidation state) methyltransferase; BHMT, betaine homocysteine methyltransferase; CBS, cystathionine-β-synthase; DHFR, dihydrofolate reductase; DMA, dimethylarsinic acid; GAA, guanidinoacetate; GAMT, guanidinoacetate *N*-methyltransferase; GNMT, glycine *N*-methyltransferase; GSS, glutathione synthetase; InAs, inorganic arsenic; MAT1A, methionine adenosyltransferase 1 (hepatic) MAT2A, methionine adenosyltransferase 2 subunit alpha (non-hepatic); MAT2B, methionine adenosyltransferase 2 subunit beta (non-hepatic); MMA, monomethylarsonic acid; MTHFD1, C-1-tetrahydrofolate synthase; MTHFR, methylene tetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate

concentrations *in vitro*, and it has been hypothesized that this is due to increased consumption of SAM via arsenic metabolism, which involves two SAM-dependent methylation reactions [60]. However, arsenic metabolism has been estimated to consume very little SAM compared with other methylation reactions [25]. In contrast, the non-essential methylation of glycine to sarcosine by *GNMT* can have large impacts on the SAM pool [59]. Thus, arsenic-induced increases in *GNMT* expression and glutathione synthesis may be alternative mechanisms by which arsenic depletes SAM concentrations.

We also observed that arsenic exposure was associated with the expression of several genes involved in epigenetic regulation, including histone methyltransferases *EZH2, MLL*, and *PRDM2*; histone deacetylase *HDAC4*; and *TET2*, one of the three enzymes that catalyzes the conversion of 5-methylcytosine to 5-hydoxymethylcytosine [61]. Arsenic exposure was positively associated with *TET2* expression among women, but not men. Interestingly, we have previously observed sex-dependent associations between arsenic and 5-hydroxymethylcytosine [17]. Arsenic-induced alterations in *TET2* expression may be one mechanism mediating this.

Of the two candidate genes involved in either tumor suppression or oncogenesis, only *TP53* was differentially expressed by arsenic exposure. Among men, but not women, arsenic was positively associated with *TP53* expression. Several previous studies, including two studies which utilized male-derived cell lines, have similarly observed that *TP53* expression is higher after arsenic exposure [62-65]. This is consistent with the fact that *TP53* is induced by cellular stressors [66].

While not statistically significant, arsenic exposure was also associated with differential methylation of several CpG sites within *AS3MT* and with lower *AS3MT* expression among men. *AS3MT* codes for the arsenic (+3 oxidation state) methyltransferase, which catalyzes both of the

methylation reactions involved in arsenic metabolism (reviewed in [67]). On average, men have a lower arsenic methylation capacity compared with women [68]. Although this has largely been attributed to sex differences in the OCM pathway [68], arsenic-induced reductions in *AS3MT* expression may be another contributing factor. However, it is important to note that our findings contrasted with an *in vitro* study, which observed that arsenic induces *AS3MT* expression in human PBMCs, although the sex of the cell donor was not specified [69].

The majority of genes that were differentially expressed by arsenic exposure were not differentially methylated. There are likely several explanations for this. First, given the smaller sample size for the methylation analyses, we had reduced statistical power to detect significant associations, particularly in sex-stratified analyses. Another possible contributing factor is the conservative nature of the Bonferroni correction. Since the average number of probes per gene is much larger for the Infinium HumanMethylation450 array compared with the HumanHT-12-v4 array, the corrected *P*-value thresholds used for the methylation analyses are much smaller than those used for the expression analyses. Furthermore, there may be additional CpG sites which are important for regulating gene expression that were not represented on the array. Finally, mRNA expression is regulated by additional mechanisms, including PTHMs and microRNAs, which are also altered by arsenic exposure [13, 18, 70]. Thus, the expression of some of our candidate genes may have been altered via these other epigenetic mechanisms.

Although our study had many strengths, including a large sample size, sex-stratified analyses, DNA methylation and mRNA expression data, and a wide range of arsenic exposures, there were several important limitations. First, the study population consisted of individuals with arsenicosis. Therefore, our findings may not be generalizable to healthy individuals. Another important limitation is that we did not have protein expression data. Some genes, such as

MTHFD1, which is involved in the OCM pathway, are altered by arsenic post-translationally (Patrick Stover, Cornell University, Personal Communication), which could not be examined in our study.

Although the use of blood-derived DNA and RNA may not be representative of other tissue types, arsenic distributes to PBMC progenitors and influences their function. For example, aquaglyceroporin 9, an arsenic transporter, is highly expressed in lymphocytes, and arsenic trioxide is used as a chemotherapeutic for acute promyelocytic leukemia [71]. Furthermore, blood DNA methylation has been used successfully as a biomarker of skin lesion risk [39], and many of our findings paralleled previous studies which had access to other target tissues, such as skin [46]. Therefore, peripheral blood appears to be an appropriate target tissue for this population.

Since we used a candidate gene approach, other important genes that may be altered by arsenic exposure were not assessed. However, a major strength of using a candidate gene approach is that it is hypothesis-driven. Additionally, by using this approach we substantially increased our statistical power, which allowed us to identify several novel targets of arsenic exposure and potential sex differences, which had not previously been examined in a large human study.

CONCLUSIONS

Previous studies have observed sex-dependent effects of arsenic on epigenetic modifications, including alterations in DNA methylation and PTHMs [12-18]. This study provides evidence that arsenic may also induce functional changes, such as alterations in mRNA expression, differentially by sex. In particular, we observed that arsenic was associated with the

expression of genes involved in the OCM pathway, epigenetic regulation, DNA repair, and tumor suppression. Dysregulation of these pathways has been implicated in arsenic toxicity. Therefore, sex-dependent perturbations in these pathways may contribute to some of the observed sex differences in susceptibility to arsenic toxicity. However, additional studies will be needed to determine if the alterations in DNA methylation and gene expression observed in this study translate to increased risks of developing arsenic-related health outcomes.

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CHAPTER SIX SUPPLEMENTAL MATERIAL

Table S1. Genes that were differentially methylated by arsenic exposure before adjusting for multiple comparisons ($P < 0.05$), either in the whole sample, in men, or in women

				Whole Sample (*n* = 400)		**Men (*n* = 212)**		**Women (*n* = 188)**	
Gene	CpG Label	# Probes	*P* Cutoff[a]	t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
Arsenic metabolism									
AS3MT	cg15744005	13	3.85 E-03	1.99	0.047*	1.85	0.065	1.06	0.290
AS3MT	cg26626287	13	3.85 E-03	2.75	0.006*	1.56	0.121	2.25	0.026*
One-carbon metabolism									
AHCY	cg07721779	27	1.85 E-03	-2.09	0.037*	-0.82	0.412	-2.13	0.035*
AHCY	cg23636941	27	1.85 E-03	1.59	0.111	2.04	0.043*	0.33	0.744
CBS	cg06118533	44	1.14 E-03	2.06	0.040*	1.32	0.188	1.58	0.115
CBS	cg09128751	44	1.14 E-03	-1.20	0.230	0.39	0.699	-1.98	0.049
CBS	cg11815682	44	1.14 E-03	2.31	0.021*	1.39	0.167	1.96	0.051
CBS	cg21006325	44	1.14 E-03	2.01	0.046*	1.23	0.219	1.58	0.116
CBS	cg22633722	44	1.14 E-03	2.11	0.035*	2.52	0.012*	0.61	0.543
DHFR	cg18200270	17	2.94 E-03	-2.02	0.044*	-0.01	0.991	-2.72	0.007*
GAMT	cg09507386	15	3.33 E-03	-1.84	0.066	-2.59	0.010*	-0.20	0.845
GAMT	cg23191024	15	3.33 E-03	1.05	0.293	2.14	0.034*	-0.63	0.530
GNMT	cg07604616	36	1.39 E-03	-1.79	0.075	-0.13	0.899	-2.34	0.020*
GNMT	cg10056627	36	1.39 E-03	2.05	0.041*	0.95	0.345	1.97	4.98 E-02*
GSS	cg13607138	13	3.85 E-03	2.32	0.021*	1.89	0.060	1.42	0.156
GSS	cg00352780	13	3.85 E-03	1.33	0.185	2.30	0.023*	-0.22	0.825
MAT1A	cg07959747	8	6.25 E-03	-1.80	0.073	-2.14	0.033*	-0.54	0.589
MAT1A	cg09936400	8	6.25 E-03	1.83	0.067	0.35	0.726	2.19	0.030*
MAT2B	cg01290068	23	2.17 E-03	0.92	0.359	1.99	0.048*	-0.60	0.546
MAT2B	cg17334359	23	2.17 E-03	-2.45	0.015*	-2.22	0.028*	-1.34	0.182
MAT2B	cg19597031	23	2.17 E-03	-1.82	0.070	0.03	0.977	-2.66	0.008*
MTHFD1	cg05143420	21	2.38 E-03	2.79	0.005*	1.24	0.215	2.59	0.010*
MTHFR	cg02978542	24	2.08 E-03	-3.08	0.002*	-0.76	0.446	-3.48	6.30 E-04*
MTHFR	cg17514528	24	2.08 E-03	-2.11	0.035	-1.19	0.237	-1.72	0.088
MTHFR	cg18276943	24	2.08 E-03	1.23	0.219	2.05	0.042*	-0.22	0.823
MTR	cg02901985	22	2.27 E-03	-1.15	0.250	1.01	0.315	-2.34	0.021*
MTR	cg17038444	22	2.27 E-03	1.91	0.056	0.30	0.761	2.18	0.031*

				Whole Sample (*n* = 400)		**Men (*n* = 212)**		**Women (*n* = 188)**	
Gene	CpG Label	# Probes	*P* Cutoff[a]	t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
One-carbon metabolism (cont.)									
MTR	cg25552893	22	2.27 E-03	2.66	0.008*	1.73	0.085	2.06	0.040*
PEMT	cg14265254	46	1.09 E-03	0.12	0.903	1.97	4.99 E-02*	-1.53	0.128
PEMT	cg15627593	46	1.09 E-03	-2.49	0.013*	-0.63	0.528	-2.73	0.007
PEMT	cg21680729	46	1.09 E-03	-1.90	0.058	-2.12	0.035*	-0.65	0.518
PEMT	cg23714707	46	1.09 E-03	2.66	0.008*	1.80	0.074	1.91	0.058
PEMT	cg26177311	46	1.09 E-03	1.97	0.049*	1.50	0.135	1.31	0.193
PEMT	cg27291501	46	1.09 E-03	-1.15	0.250	1.27	0.205	-2.50	0.013
Epigenetic regulation									
DNMT1	cg02762710	22	2.27 E-03	1.67	0.095	0.06	0.955	2.13	0.035*
DNMT3A	cg08316074	79	6.33 E-04	2.75	0.006*	1.41	0.161	2.37	0.019*
DNMT3A	cg10525105	79	6.33 E-04	0.94	0.346	-1.18	0.239	2.38	0.018*
DNMT3A	cg11354105	79	6.33 E-04	1.58	0.115	0.10	0.923	2.16	0.032*
DNMT3A	cg23903708	79	6.33 E-04	-2.38	0.018*	-2.70	0.008*	-0.76	0.449
DNMT3B	cg22052056	20	2.50 E-03	-2.36	0.019*	-0.72	0.475	-2.61	0.010*
DOT1L	cg03336268	53	9.40 E-04	1.31	0.191	2.06	0.041*	-0.06	0.953
DOT1L	cg04173586	53	9.40 E-04	-2.87	0.004*	-1.67	0.096	-2.37	0.019*
DOT1L	cg04248042	53	9.40 E-04	-1.20	0.231	0.46	0.645	-1.98	4.96 E-02*
EHMT1	cg01219549	81	6.20 E-04	2.23	0.026*	-0.06	0.949	3.09	0.002*
EHMT1	cg10615711	81	6.20 E-04	-0.21	0.833	-2.29	0.023*	1.81	0.072
EHMT1	cg13434216	81	6.20 E-04	-2.43	0.016*	-1.58	0.116	-1.83	0.069
EHMT1	cg13555335	81	6.20 E-04	-1.27	0.206	-2.57	0.011	0.56	0.578
EHMT1	cg13791668	81	6.20 E-04	1.45	0.149	-0.22	0.823	2.22	0.027*
EHMT1	cg13922757	81	6.20 E-04	-2.63	0.009*	-0.96	0.336	-2.67	0.008*
EHMT1	cg14156842	81	6.20 E-04	1.31	0.191	-0.24	0.810	1.98	0.049*
EHMT1	cg14459021	81	6.20 E-04	2.22	0.027*	2.01	0.046*	1.17	0.244
EHMT1	cg14469972	81	6.20 E-04	1.43	0.153	0.02	0.986	2.02	0.045*
EHMT1	cg17942750	81	6.20 E-04	2.15	0.032*	1.18	0.240	1.80	0.074
EHMT1	cg18819574	81	6.20 E-04	1.15	0.249	-0.51	0.613	2.04	0.043*
EHMT2	cg01168115	177	2.80 E-04	2.56	0.011*	1.94	0.054	1.69	0.093
EHMT2	cg02760218	177	2.80 E-04	2.35	0.019*	0.77	0.442	2.63	0.009*

				Whole Sample (*n* = 400)		**Men (*n* = 212)**		**Women (*n* = 188)**	
Gene	CpG Label	# Probes	*P* Cutoff[a]	t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
Epigenetic regulation (cont.)									
EHMT2	cg06824988	177	2.80 E-04	2.03	0.043*	1.27	0.205	1.55	0.123
EHMT2	cg07829740	177	2.80 E-04	2.79	0.005*	2.06	0.041*	1.85	0.066
EHMT2	cg09277816	177	2.80 E-04	-1.26	0.209	0.62	0.535	-2.28	0.024*
EHMT2	cg13613346	177	2.80 E-04	-2.04	0.042*	-2.11	0.036*	-0.91	0.363
EHMT2	cg19577206	177	2.80 E-04	-1.86	0.064	-2.81	0.006	0.05	0.961
EHMT2	cg21141346	177	2.80 E-04	1.67	0.096	-0.45	0.652	2.71	0.007*
EHMT2	cg21252609	177	2.80 E-04	1.24	0.215	2.02	0.044	-0.08	0.934
EHMT2	cg21786114	177	2.80 E-04	-2.04	0.042*	-0.47	0.638	-2.27	0.024*
EHMT2	cg22397673	177	2.80 E-04	1.54	0.125	-0.27	0.788	2.36	0.019
EHMT2	cg22706070	177	2.80 E-04	2.25	0.025*	2.22	0.027*	0.98	0.327
EHMT2	cg24899451	177	2.80 E-04	0.74	0.457	2.11	0.036*	-0.99	0.323
EHMT2	cg25633383	177	2.80 E-04	2.10	0.036*	2.34	0.020*	0.83	0.409
EHMT2	cg25644015	177	2.80 E-04	2.09	0.037*	1.71	0.089	1.32	0.190
HDAC4	cg00116699	427	1.20 E-04	-1.85	0.065	-2.05	0.042*	-0.60	0.552
HDAC4	cg00731459	427	1.20 E-04	2.11	0.035*	2.55	0.011*	0.50	0.619
HDAC4	cg01114124	427	1.20 E-04	-1.38	0.170	-2.24	0.026*	0.09	0.930
HDAC4	cg01786275	427	1.20 E-04	1.48	0.140	0.11	0.912	2.05	0.042*
HDAC4	cg01790646	427	1.20 E-04	-0.78	0.436	1.26	0.209	-2.23	0.027*
HDAC4	cg01974660	427	1.20 E-04	-0.86	0.391	1.17	0.242	-2.22	0.028*
HDAC4	cg03475776	427	1.20 E-04	-1.36	0.175	0.95	0.341	-2.78	0.006*
HDAC4	cg04346861	427	1.20 E-04	2.29	0.023*	0.91	0.365	2.22	0.027*
HDAC4	cg04521026	427	1.20 E-04	-2.55	0.011*	-1.33	0.185	-2.23	0.027*
HDAC4	cg05114739	427	1.20 E-04	2.20	0.028*	1.93	0.055	1.18	0.238
HDAC4	cg05903736	427	1.20 E-04	-2.06	0.040*	-0.70	0.484	-2.16	0.032*
HDAC4	cg06223736	427	1.20 E-04	-1.99	0.047*	-0.65	0.519	-2.10	0.037*
HDAC4	cg06533788	427	1.20 E-04	1.42	0.155	-0.29	0.769	2.26	0.025*
HDAC4	cg06855182	427	1.20 E-04	1.48	0.139	-0.31	0.760	2.38	0.018*
HDAC4	cg07150777	427	1.20 E-04	2.22	0.027*	0.23	0.816	3.04	0.003*
HDAC4	cg07215298	427	1.20 E-04	-3.78	1.8 E-04*	-3.62	3.7 E-04*	-1.81	0.072
HDAC4	cg07554496	427	1.20 E-04	2.10	0.036*	0.61	0.541	2.31	0.022*

				Whole Sample (*n* = 400)		**Men (*n* = 212)**		**Women (*n* = 188)**	
Gene	CpG Label	# Probes	*P* Cutoff[a]	t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
Epigenetic regulation (cont.)									
HDAC4	cg07673080	427	1.20 E-04	1.99	0.048*	2.84	0.005*	0.18	0.857
HDAC4	cg08107308	427	1.20 E-04	-2.59	0.010*	-1.67	0.097	-1.97	0.050
HDAC4	cg09234543	427	1.20 E-04	1.61	0.108	-0.32	0.746	2.55	0.012*
HDAC4	cg09669931	427	1.20 E-04	2.79	0.006*	1.12	0.265	2.86	0.005*
HDAC4	cg09799039	427	1.20 E-04	2.72	0.007*	2.15	0.033*	1.70	0.090
HDAC4	cg10045864	427	1.20 E-04	-2.20	0.028*	-1.76	0.080	-1.32	0.187
HDAC4	cg10546410	427	1.20 E-04	1.34	0.182	2.00	0.047*	0.09	0.928
HDAC4	cg10639368	427	1.20 E-04	2.03	0.043*	3.18	0.002*	-0.02	0.981
HDAC4	cg10973720	427	1.20 E-04	0.95	0.344	-1.09	0.276	2.46	0.015*
HDAC4	cg11349429	427	1.20 E-04	1.21	0.227	-0.68	0.496	2.24	0.026*
HDAC4	cg11534215	427	1.20 E-04	1.08	0.281	-1.13	0.260*	2.51	0.013*
HDAC4	cg12793681	427	1.20 E-04	2.18	0.030*	1.19	0.234	1.88	0.062
HDAC4	cg13278833	427	1.20 E-04	-2.12	0.035*	-0.81	0.419	-2.10	0.037*
HDAC4	cg14780600	427	1.20 E-04	1.08	0.281	-0.77	0.444	2.28	0.024*
HDAC4	cg15142485	427	1.20 E-04	-0.65	0.515	1.93	0.055	-2.72	0.007*
HDAC4	cg15929228	427	1.20 E-04	1.52	0.130	2.13	0.034*	0.18	0.854
HDAC4	cg16325984	427	1.20 E-04	1.87	0.063	0.35	0.724	2.22	0.028*
HDAC4	cg16468346	427	1.20 E-04	-2.02	0.044*	-2.86	0.005*	-0.10	0.924
HDAC4	cg17156828	427	1.20 E-04	1.37	0.172	-0.75	0.452	2.55	0.012*
HDAC4	cg17285931	427	1.20 E-04	-2.07	0.039*	-1.49	0.137	-1.42	0.157
HDAC4	cg19367293	427	1.20 E-04	2.00	0.046*	1.29	0.200	1.55	0.123
HDAC4	cg20854286	427	1.20 E-04	1.10	0.311	-1.15	0.251	2.29	0.023*
HDAC4	cg20859099	427	1.20 E-04	2.17	0.031*	0.66	0.507	2.31	0.022*
HDAC4	cg21190228	427	1.20 E-04	-2.18	0.029*	-0.75	0.452	-2.26	0.025*
HDAC4	cg23870168	427	1.20 E-04	1.69	0.093	2.07	0.040*	0.33	0.740
HDAC4	cg25236416	427	1.20 E-04	2.79	0.006*	1.43	0.153	2.39	0.018*
HDAC4	cg25521439	427	1.20 E-04	-1.32	0.189	0.93	0.354	-2.68	0.008*
HDAC4	cg26673264	427	1.20 E-04	-2.24	0.025*	-0.80	0.425	-2.25	0.026*
HDAC4	cg26913798	427	1.20 E-04	1.97	4.98 E-02*	1.41	0.161	1.37	0.174
HDAC4	cg26975040	427	1.20 E-04	-1.23	0.218	0.46	0.647	-2.00	0.046*

				Whole Sample (*n* = 400)		**Men (*n* = 212)**		**Women (*n* = 188)**	
Gene	CpG Label	# Probes	*P* Cutoff[a]	t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
Epigenetic regulation (cont.)									
HDAC4	cg27074582	427	1.20 E-04	-0.97	0.335	1.11	0.269	-2.45	0.015*
HDAC4	cg27144223	427	1.20 E-04	-1.87	0.063	-0.18	0.856	-2.53	0.012*
HDAC5	cg08531489	22	2.27 E-03	-1.52	0.128	0.45	0.650	-2.44	0.016
HDAC5	cg11049075	22	2.27 E-03	-0.90	0.368	0.87	0.384	-2.20	0.029*
HDAC5	cg25334369	22	2.27 E-03	1.37	0.171	2.03	0.043*	-0.02	0.981
KDM2B	cg04682193	84	6.00 E-04	-1.98	0.048*	-0.98	0.329	-1.75	0.082
KDM2B	cg09411874	84	6.00 E-04	1.98	0.048*	1.53	0.129	1.24	0.218
KDM2B	cg10507156	84	6.00 E-04	1.58	0.116	-0.18	0.858	2.31	0.022*
KDM2B	cg16655291	84	6.00 E-04	2.25	0.025*	1.28	0.204	1.75	0.082
KDM2B	cg23877401	84	6.00 E-04	-2.57	0.011*	-0.74	0.460	-2.79	0.006*
KDM2B	cg26509318	84	6.00 E-04	-1.34	0.182	0.41	0.683	-2.07	0.040*
KDM2B	cg26995224	84	6.00 E-04	2.17	0.031*	1.81	0.072	1.29	0.200
KDM4B	cg02302043	118	4.20 E-04	-1.32	0.187	-2.15	0.033*	0.19	0.847
KDM4B	cg03315649	118	4.20 E-04	-3.67	2.8 E-04*	-2.14	0.033*	-3.01	0.003*
KDM4B	cg05877783	118	4.20 E-04	-1.07	0.284	-2.15	0.032*	0.64	0.520
KDM4B	cg09527670	118	4.20 E-04	2.03	0.043*	1.62	0.107	1.28	0.203
KDM4B	cg11645724	118	4.20 E-04	-2.08	0.038*	-1.58	0.116	-1.35	0.178
KDM4B	cg18362538	118	4.20 E-04	-1.22	0.224	-2.23	0.027*	0.48	0.634
KDM4B	cg20347343	118	4.20 E-04	1.18	0.238	2.31	0.022*	-0.53	0.595
KDM4B	cg22960833	118	4.20 E-04	-1.10	0.270	-2.19	0.030*	0.52	0.604
MLL	cg02871053	18	2.78 E-03	-2.00	0.047*	-1.59	0.113	-1.22	0.226
NSD1	cg10821304	28	1.79 E-03	-1.80	0.073	-2.14	0.033	-0.46	0.649
NSD1	cg17493885	28	1.79 E-03	1.68	0.094	0.07	0.948	1.99	0.048*
NSD1	cg19731612	28	1.79 E-03	2.00	0.046*	-0.31	0.754	2.55	0.012*
PRDM2	cg06604289	65	7.70 E-04	-2.14	0.033*	-0.77	0.441	-2.22	0.028*
PRDM2	cg06991148	65	7.70 E-04	2.19	0.029*	0.35	0.725	2.82	0.005*
PRDM2	cg10444683	65	7.70 E-04	2.28	0.023*	0.69	0.492	2.38	0.018*
PRDM2	cg21605566	65	7.70 E-04	1.97	4.95 E-02*	1.42	0.158	1.36	0.175
TET1	cg05400741	30	1.67 E-03	1.53	0.126	-0.03	0.976	2.12	0.035*
TET1	cg12548760	30	1.67 E-03	1.57	0.118	0.20	0.840	1.99	0.048*

				Whole Sample (*n* = 400)		**Men (*n* = 212)**		**Women (*n* = 188)**	
Gene	CpG Label	# Probes	*P* Cutoff[a]	t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
Epigenetic regulation (cont.)									
TET2	cg14330655	21	2.38 E-03	2.25	0.025*	2.07	0.040*	1.09	0.276
TET3	cg25299214	14	3.57 E-03	0.99	0.325	-0.84	0.399	2.01	0.046*
Tumor suppression									
EGFR	cg20706768	58	8.60 E-04	2.07	0.039*	1.12	0.265	1.78	0.077
EGFR	cg10550611	58	8.60 E-04	-2.19	0.029*	-2.72	0.007*	-0.55	0.582
EGFR	cg16751451	58	8.60 E-04	1.35	0.179	-0.22	0.824	2.05	0.042*
EGFR	cg21901928	58	8.60 E-04	1.84	0.066	0.10	0.922	2.54	0.012*
EGFR	cg27637738	58	8.60 E-04	-1.83	0.067	-2.21	0.028*	-0.45	0.653
TP53	cg01620719	39	1.28 E-03	-2.18	0.030*	-1.54	0.124	-1.54	0.124
TP53	cg05479194	39	1.28 E-03	2.42	0.016*	2.00	0.046*	1.47	0.142
TP53	cg12373934	39	1.28 E-03	-0.56	0.579	1.45	0.148	-2.06	0.040*
DNA repair									
MSH2	cg14282180	14	3.57 E-03	-1.82	0.069	0.19	0.848	-2.65	0.009*
MSH2	cg19180827	14	3.57 E-03	-0.95	0.344	0.87	0.384	-2.10	0.037*
MSH2	cg25746226	14	3.57 E-03	-1.60	0.111	0.67	0.502	-2.69	0.008*
PARP1	cg17127702	19	2.63 E-03	-0.22	0.827	1.89	0.060	-2.35	0.020*
PCNA	cg01195281	26	1.92 E-03	1.24	0.216	-0.61	0.544	2.29	0.023*
PCNA	cg01511104	26	1.92 E-03	2.05	0.041*	1.85	0.065	1.12	0.264
PCNA	cg09011324	26	1.92 E-03	-2.64	0.009*	-0.14	0.890	-3.66	3.3 E-04*
PCNA	cg22960971	26	1.92 E-03	-3.49	5.30 E-04*	-1.92	0.056	-2.98	0.003*
XRCC1	cg02455501	12	4.17 E-03	-2.47	0.014*	-1.73	0.085	-1.74	0.083
XRCC1	cg15107336	12	4.17 E-03	-1.84	0.066	-0.50	0.614	-2.10	0.037*
XRCC2	cg22488067	13	3.85 E-03	1.34	0.182	2.00	0.047*	-0.09	0.929
XRCC2	cg05898482	13	3.85 E-03	1.04	0.297	2.65	0.009*	-0.79	0.429
XRCC3	cg05182418	37	1.35 E-03	-0.38	0.702	-2.73	0.007*	1.88	0.062
XRCC3	cg12798040	37	1.35 E-03	-2.78	0.006*	-2.78	0.006*	-1.29	0.198
XRCC3	cg13255208	37	1.35 E-03	-1.59	0.113	0.55	0.584	-3.02	0.003*
XRCC3	cg27077050	37	1.35 E-03	-1.48	0.140	-0.14	0.890	-1.98	0.049*
XRCC4	cg07357445	25	0.002	1.41	0.160	-0.32	0.750	2.15	0.033*
XRCC4	cg15676060	25	0.002	0.45	0.655	2.02	0.045*	-1.31	0.193

				Whole Sample (*n* = 400)		**Men (*n* = 212)**		**Women (*n* = 188)**	
Gene	CpG Label	# Probes	*P* Cutoff[a]	t-statistic[b]	*P*	t-statistic[b]	*P*	t-statistic[b]	*P*
DNA repair (cont.)									
XRCC4	cg19756313	25	0.002	-1.33	0.186	0.19	0.846	-1.99	0.048*
XRCC4	cg25112586	25	0.002	-1.52	0.130	0.05	0.959	-2.01	0.045*
XRCC5	cg05998850	20	2.50 E-03	1.12	0.265	-1.75	0.082	3.14	0.002*
XRCC6	ch22757911F	20	2.50 E-03	2.04	0.042*	2.30	0.023*	0.66	0.510

Abbreviations used: CpG, cytosine guanine dinucleotide

[a]*P* cutoff is based on an alpha of 0.05, which was adjusted using the Bonferroni method to account for the number of probes representing the gene on the microarray
[b]t-statistic and corresponding *P*-value are from linear regression models. Whole sample analyses were adjusted for sex and age. Sex-stratified analyses were adjusted for age.
**P* value was statistically significant before adjusting for multiple comparisons ($P < 0.05$)

Table S2. Genes that were differentially expressed by arsenic exposure before adjusting for multiple comparisons ($P < 0.05$) either in the whole sample, in men, or in women

				Whole Sample (*n* = 1799)		**Males (*n* = 991)**		**Females (*n* = 801)**	
Gene	Probe	# Probes	*P* Cutoff[a]	β[b]	*P*	β[b]	*P*	β[b]	*P*
One-carbon metabolism									
AHCY	ILMN_1657862	1	0.050	2.62 E-05	0.110	4.27 E-05	0.036*	2.14 E-06	0.936
CBS	ILMN_1804735	1	0.050	2.28 E-05	0.035*	1.06 E-05	0.448	4.05 E-05	0.019*
DHFR	ILMN_1759872	2	0.025	6.59 E-05	0.008*	7.01 E-05	0.022*	6.19 E-05	0.133
DNMT3A	ILMN_1654945	3	0.017	1.84 E-05	0.109	3.11 E-05	0.031*	3.18 E-07	0.986
GAMT	ILMN_1756469	3	0.017	-3.98 E-05	0.001*	-2.37 E-05	0.133	-6.49 E-05	9.60 E-04*
GAMT	ILMN_1794595	3	0.017	-1.89 E-05	0.091	-9.50 E-07	0.947	-4.72 E-05	0.008*
GNMT	ILMN_1736238	1	0.050	3.62 E-06	0.578	-7.62 E-06	0.350	2.12 E-05	0.044*
GSS	ILMN_1683462	1	0.050	3.66 E-05	1.08 E-04*	4.04 E-05	8.64 E-04*	3.22 E-05	0.033*
MAT2B	ILMN_1811367	3	0.017	-3.59 E-05	0.037*	-2.65 E-05	0.247	-5.06 E-05	0.056
MTR	ILMN_1670801	1	0.050	-2.81 E-05	0.022*	-3.24 E-05	0.043*	-2.17 E-05	0.262
Epigenetic regulation									
EZH2	ILMN_1708105	3	0.017	4.09 E-05	1.46 E-05*	3.29 E-05	0.007*	5.18 E-05	5.28 E-04*
EZH2	ILMN_2364529	3	0.017	3.58 E-05	1.3 E-04*	2.86 E-05	0.015*	4.53 E-05	0.003*
EZH2	ILMN_1652913	3	0.017	3.17 E-05	0.003*	2.72 E-05	0.047*	3.83 E-05	0.028*
HDAC4	ILMN_1764396	1	0.050	-2.32 E-05	0.071	-3.78 E-05	0.017*	-8.31 E-07	0.969
MLL	ILMN_1668683	1	0.050	-6.59 E-05	6.80 E-08*	-5.45 E-05	4.92 E-04*	-8.13 E-05	2.86 E-05*
NSD1	ILMN_1707175	2	0.025	-2.05 E-05	0.025*	-2.14 E-05	0.078	1.90 E-05	0.182
PRDM2	ILMN_2258543	4	0.013	-1.71 E-05	0.029*	-1.70 E-05	0.085	-1.73 E-05	0.172
PRDM2	ILMN_1652992	4	0.013	-2.03 E-05	0.002*	-1.98 E-05	0.026*	-2.07 E-05	0.038*
TET2	ILMN_1788818	1	0.050	2.18 E-05	0.008*	-3.19 E-06	0.757	5.94 E-05	8.23 E-06*

				Whole Sample (*n* = 1799)		Males (*n* = 991)		Females (*n* = 801)	
Gene	Probe	# Probes	*P* Cutoff[a]	β[b]	*P*	β[b]	*P*	β[b]	*P*
Tumor suppression									
EGFR	ILMN_1798975	4	0.013	9.20 E-06	0.073	1.51 E-05	0.021*	3.86 E-07	0.962
TP53	ILMN_1779356	1	0.050	2.47 E-05	0.075	4.93 E-05	0.006*	-9.68 E-06	0.660
DNA Repair									
MSH2	ILMN_1737413	2	0.025	-2.42 E-05	0.016*	-2.05 E-05	0.116	-3.08 E-05	0.051
POLB	ILMN_1767894	1	0.050	5.01 E-05	3.77 E-05*	3.85 E-05	0.016*	6.99 E-05	2.15 E-04*
XRCC3	ILMN_1696266	1	0.050	9.88 E-06	0.420	3.13 E-05	0.047*	-2.18 E-05	0.262
XRCC5	ILMN_2105983	1	0.050	-2.84 E-05	0.014*	-9.92 E-06	0.509	-5.47 E-05	0.003*
XRCC6	ILMN_1743097	2	0.025	-4.61 E-05	1.96 E-04*	-3.60 E-05	0.022*	-6.24 E-05	0.002*

[a] *P* cutoff is based on an alpha of 0.05, which was adjusted using the Bonferroni method to account for the number of probes representing the gene on the microarray

[b] β and corresponding *P*-value are from linear regression models. Whole sample analyses were adjusted for sex and age. Sex-stratified analyses were adjusted for age

**P* value was statistically significant before correcting for multiple comparisons ($P < 0.05$)

CHAPTER SEVEN

Folic acid supplementation and sex-specific associations between one-carbon metabolism indices and histone modifications in arsenic-exposed Bangladeshi adults

Caitlin G. Howe[1], Xinhua Liu[2], Megan N. Hall[3], Vesna Ilievski[1], Marie A. Caudill[4], Olga Malysheva[4], Angela M. Lomax-Luu[1], Faruque Parvez[1], Abu B. Siddique[5], Hasan Shahriar[5], Mohammad N. Uddin[5], Tariqul Islam[5], Joseph H. Graziano[1], Max Costa[6], Mary V. Gamble[1]

Affiliations: Departments of [1]Environmental Health Sciences, [2]Biostatistics, [3]Epidemiology, Mailman School of Public Health, New York, New York; [4]Division of Nutritional Sciences, Cornell University, Ithaca, New York; [5]Columbia University Arsenic Project in Bangladesh, Dhaka, Bangladesh; [6]Department of Environmental Medicine, NYU Langone Medical Center, New York University, New York, New York

ABSTRACT:

Background: Posttranslational histone modifications (PTHMs) are altered by arsenic, an environmental carcinogen. PTHMs are also influenced by nutritional methyl donors involved in one-carbon metabolism (OCM), which may protect against epigenetic dysregulation.

Methods: We measured global levels of three PTHMs, which are dysregulated in cancers (H3K36me2, H3K36me3, H3K79me2), in peripheral blood mononuclear cells (PBMCs) collected from 324 participants enrolled in the Folic Acid and Creatine Trial, a randomized trial in arsenic-exposed Bangladeshi adults. Sex-specific associations between blood OCM indices (folate, cobalamin, choline, betaine, homocysteine) and PTHMs were examined at baseline using regression models. We also evaluated the effects of FA supplementation (400 μg/day for 12 weeks), compared with placebo, on PTHMs.

Results: Associations between choline and H3K36me2 differed by sex ($P_{interaction} < 0.01$). This was also true for associations between cobalamin and H3K79me2 ($P_{interaction} < 0.05$). Among men, plasma choline was positively associated with H3K36me2 ($P < 0.01$), and among women, plasma cobalamin was positively associated with H3K79me2 ($P < 0.01$). FA supplementation did not alter any of the PTHMs examined ($P > 0.05$).

Conclusion: OCM indices may influence PTHMs in a sex-dependent manner, and FA supplementation, at this dose and duration, does not alter PTHMs in PBMCs.

Impact: This is the first study to examine the influences of OCM indices on PTHMs in a population that may have increased susceptibility to cancer development due to widespread exposure to arsenic-contaminated drinking water and a high prevalence of hyperhomocysteinemia.

INTRODUCTION:

More than 150 different methyltransferases, including DNA methyltransferases and lysine histone methyltransferases, depend on methyl donations from *S*-adenosylmethionine (SAM) [1]. Synthesis of SAM via one-carbon metabolism (OCM) involves the remethylation of homocysteine (Hcys) to methionine, which requires nutritional methyl donors and cofactors, such as folate, cobalamin, choline, and betaine. There are important sex differences in the OCM pathway. For example, plasma Hcys concentrations are higher in men, and there is evidence that this may be due to both higher methyl demand for creatine synthesis as a result of greater muscle mass [2] and also lower remethylation and transmethylation rates among men [3]. Circulating concentrations of folate, cobalamin, and choline also differ by sex [4, 5].

Hyperhomocysteinemia (HHcys) and insufficient intake of nutritional methyl donors have been implicated in the development of human cancers [6]. Studies in rodents have demonstrated that this may be mediated by alterations in epigenetic modifications [7, 8], including PTHMs [9, 10]. Nutritional methyl donors have also been shown to modify, or buffer against, epigenetic dysregulation caused by environmental toxicants. For example, mice exposed *in utero* to the endocrine disruptor bisphenol A have reduced levels of DNA methylation in several tissues, but this phenotype can be prevented with maternal supplementation with folic acid (FA), cobalamin, choline, and betaine [11]. Similarly, Bangladeshi adults exposed chronically to arsenic, a human carcinogen, have higher global levels of DNA methylation in leukocytes, but only in those that are folate sufficient (plasma folate >9 nmol/L) [12]; this may be a protective compensatory mechanism, as leukocyte hypomethylation and folate deficiency are both risk factors for arsenic-induced skin lesions [13]. Concurrent exposure to arsenic and a methyl deficient diet also alters global DNA methylation in the mouse liver in a sex-dependent

manner [14]. We, and others, have previously observed sex-specific effects of arsenic exposure on global levels of DNA methylation and PTHMs in human populations [15-18]. However, the relationships between OCM indices and PTHMs, and potential differences by sex, have not been investigated.

We therefore examined sex-specific associations between OCM indices and global levels of PTHMs in a population in Bangladesh that may have increased susceptibility to cancer development due to a high prevalence of HHcys and widespread exposure to arsenic-contaminated drinking water. We also examined the effect of folic acid (FA) supplementation (400 µg/day for 12 weeks) on PTHMs. We selected three PTHMs (histone H3 lysine 36 di- and tri-methylation (H3K36me2 and H3K36me3, respectively), and histone H3 lysine 79 di-methylation (H3K79me2)), which are dysregulated in several types of cancer [19-24] and are altered by arsenic and/or nutritional methyl donors in experimental models [25-28]. PTHMs were measured in peripheral blood mononuclear cells (PBMCs) collected from participants in the Folic Acid and Creatine Trial (FACT). FACT is a randomized clinical trial that was originally designed to examine whether FA and/or creatine supplementation can be used as therapeutic approaches to reduce blood arsenic concentrations; the primary findings of this trial have been published [29].

STUDY PARTICIPANTS AND METHODS:

Region and Participants

Participants for the FACT study were recruited from the Health Effects of Arsenic Longitudinal Study, a prospective cohort study that initially recruited 11,746 adults living in a 25 km^2 region in Araihazar, Bangladesh [30]. FACT is a double-blind randomized, placebo-

controlled trial [29]. FACT participants were between the ages of 20 and 65 and had been drinking from household wells with water arsenic ≥50 μg/L, the Bangladesh standard for safe drinking water. Exclusion criteria included: pregnancy, nutritional supplement use, and known health problems, including cancers. Informed consent was obtained by Bangladeshi field staff physicians, and this study was approved by the Institutional Review Board of Columbia University Medical Center and the Bangladesh Medical Research Council.

Study Design

As described previously [29], FACT participants (*n* = 622) were randomized to one of five treatment arms: placebo (*n* = 104), 400 μg FA/day (*n* = 156), 800 μg FA/day (*n* = 154), 3 g creatine/day (*n* = 104), and 3 g creatine + 400 μg FA/day (*n* = 104) (**Supplemental Material, Figure S1**). Due to ethical considerations, all participants received arsenic-removal water filters (READ-F filter, Brota Services International, Bangladesh) at baseline to be used during the 24 week study period and thereafter.

Whole blood samples were collected from participants at baseline, week 12, and week 24; sample collection and handling have been described previously [15, 29]. For the current study, we used histones isolated from baseline (i.e., pre-intervention)-collected PBMCs from a subset of participants with all necessary biological samples and complete data for relevant covariates (*n* = 324), as well as available PBMCs collected at week 12 from participants in the placebo (*n* = 60) and 400 μg FA (*n* = 107) treatment arms (Supplemental Material, Figure S1). The 400 μg FA dose was selected based on the U.S. recommended dietary allowance for adults [31].

Folate and Cobalamin

Plasma folate and cobalamin were measured by radio-protein-binding assay (SimulTRAC-SNB, MP Biomedicals). The intra- and inter-assay CVs were 5% and 13%, respectively, for folate and 6% and 17%, respectively, for cobalamin. Folate in whole blood hemolysate was also measured by radio-protein-binding assay (SimulTRAC-S, MP Biomedicals) in participants from the placebo, 400 µg FA/day, and 800 µg FA/day treatment arms, as described previously [29]; red blood cell (RBC) folate was calculated by dividing these measures by [%hematocrit/100]. The intra- and inter-assay CVs for RBC folate were 4% and 9%, respectively.

Plasma Choline and Betaine

Plasma choline and betaine concentrations were measured by LC-MS/MS, using the method of Holm et al. [32], with some modifications, as described previously [33]. The intra- and inter-assay CVs for plasma choline were 2.2% and 5.8%, respectively, and were 2.5% and 5.6%, respectively, for plasma betaine.

Plasma Homocysteine

Plasma total homocysteine (Hcys) was measured by HPLC with fluorescence detection, based on a method described by Pfeiffer et al. [34]. The intra- and inter-assay CVs were 5% and 7%, respectively.

Blood Arsenic and Selenium

Total blood arsenic and selenium (bSe) concentrations were measured using a Perkin-Elmer Elan DRC II ICP-MS equipped with an AS10+ autosampler based on a previously

described method [35]. The intra- and inter-assay CVs for arsenic were 2.7% and 5.7%, respectively, and were 1.5% and 4.6%, respectively, for bSe.

Urinary Creatinine

Urinary creatinine (uCr) was measured by a method based on the Jaffe reaction [36], and the intra- and inter-assay CVs were 1.3% and 2.9%, respectively

Histone Isolation

Histones were isolated from PBMCs by acid extraction, as described previously [15, 16]. Isolated histones were diluted in 4 M urea, and aliquots were stored at -80ºC.

H3K36me2, H3K36me3, H3K79me2

Although we previously identified a specific cleavage product of histone H3 which interferes with the measurement of downstream PTHMs, H3K36me2, H3K36me3, and H3K79me2 are not impacted by H3 cleavage [37]. These PTHMs were measured by sandwich ELISA [16]. The intra- and inter-assay CVs, respectively, for each ELISA method were as follows: H3K36me2: 3.4% and 9.6%, H3K36me3: 4.9% and 11.9%, and H3K79me2: 7.1% and 7.0%. Since there were limited histone aliquots for the final assays, and since samples with poor reproducibility were excluded, final sample sizes for H3K36me2 (n = 318) and H3K36me3 (n = 306) were smaller than the final sample size for H3K79me2 (n = 321).

Statistical methods

Differences in continuous and categorical variables between men and women and also between participants with and without PTHM measures and with and without RBC folate measures were assessed using Wilcoxon rank-sum and Chi-square tests, respectively.

Transformations were applied to variables with skewed distributions to stabilize variances for parametric model assumptions and to reduce the influence of extreme values. Natural log-transformations were applied to H3K36me3, H3K79me2, bSe, RBC folate, and plasma folate, choline, betaine, cobalamin, and Hcys. An inverse transformation (1/x) was applied to H3K36me2.

Due to the distribution of H3K36me2, a generalized linear model with an inverse-link function (which effectively back-transforms the inverse-transformed H3K36me2 variable) was used to examine associations between the log-transformed OCM indices and the harmonic mean of H3K36me2. Linear models were used to examine associations between log-transformed OCM indices and log(H3K36me3) and log(H3K79me2). Plasma folate, choline, betaine, cobalamin, and Hcys were included simultaneously in models. Alternative models replacing plasma folate with RBC folate were applied to the subset of participants with RBC folate measures (n = 250). Models were run separately by sex, and the Wald test was used to determine if associations between each OCM variable and PTHM differed by sex. Models were additionally adjusted for hypothesized confounders of the relationships between OCM indices and PTHMs, and any variables that were associated with PTHMs in bivariate analyses. Age, education, TV ownership, log(bSe), and cigarette smoking (for analyses of H3K36me3 and H3K79me2 in men) were included as covariates in final models. All covariates were included as continuous variables, except for TV ownership, cigarette smoking status, and education; the latter was included as a binary variable (education >5 y vs. ≤5 y) because many participants had 0 or few years of education. In sensitivity analyses, we also examined models that 1) were additionally adjusted for log(BMI) and log-transformed arsenic measures and 2) evaluated each of the OCM indices individually. The difference in the within-person change for each PTHM between the two

treatment arms (400 µg FA vs. placebo) was examined using the Wilcoxon rank-sum test. In exploratory analyses, we also examined potential differences in the effects of FA on PTHMs separately by sex. The significance level was set at $P < 0.05$ for all statistical tests. Analyses were conducted using SAS (version 9.3, Cary, NC) and R (version 3.1.3).

RESULTS:

General Characteristics, Nutritional Indices, and PTHMs

General characteristics of the study participants have been described previously [29], and are presented separately by sex in **Supplemental Material, Table S1**. Participants were between 24 and 54 years old with a median BMI of 19.2 kg/m^2. Median plasma choline and betaine concentrations were 11.0 and 42.8 µmol/L, respectively. Approximately, 23% of participants were folate deficient (plasma folate <9 nmol/L [38]) and 24% were cobalamin deficient (plasma cobalamin <151 pmol/L [38]). The prevalence of HHcys (plasma Hcys ≥13 µmol/L) was 40.7%. Compared with women, men in the study sample were generally older; were less likely to own a TV (an indicator of socioeconomic status in this population); had lower BMIs; had higher bAs, choline, betaine, and Hcys concentrations; and were more likely to be folate deficient and to have HHcys. Men were also much more likely to have ever smoked cigarettes. The prevalence of ever smoking was 56.2% in men compared with 1.2% in women. Baseline measures of PTHMs did not differ significantly between men and women.

Study participants with PTHM measures were generally comparable to the rest of the FACT participants, but were slightly older, had lower plasma folate, betaine, and choline concentrations, and were less likely to own TVs (**Supplemental Material, Table S2**). The subset of participants with RBC folate measures had lower plasma cobalamin concentrations and

uCr concentrations and were more likely to own TVs, but were otherwise comparable to FACT participants who did not have RBC folate measures (**Supplemental Material, Table S3**).

Sex-Specific Associations between OCM Indices and PTHMs

Sex-specific associations between OCM indices and PTHMs are shown in **Figure 1**. Alternative models, which replaced plasma folate with RBC folate, are shown in **Supplemental Material, Figure S2**. Plasma betaine was excluded from final models as it was not associated with any of the PTHMs in men or women after adjusting for plasma choline, and its addition to models did not alter coefficients for any of the other OCM indices.

H3K36me2

Log(RBC folate) was negatively and significantly associated with H3K36me2 among men (β: -0.23; 95% CI: -0.41, -0.05; $P = 0.01$). There was a similar trend among women (β: -0.08; 95% CI: -0.21, 0.04; $P = 0.19$). Neither log(plasma folate) nor log(cobalamin) was associated with H3K36me2 in men or women. Associations between log(choline) and H3K36me2 differed by sex ($P < 0.01$). Log(choline) was positively associated with H3K36me2 in men (β: 0.39; 95% CI: 0.11, 0.66; $P < 0.01$), but not women (β: -0.11; 95% CI: -0.36, 0.14; $P = 0.40$). There was also a negative association between log(Hcys) and H3K36me2 among men (β: -0.19; 95% CI: -0.30, -0.07; $P < 0.01$), but not women (β: 0.00; 95% CI: -0.16, 0.15; $P = 0.97$).

H3K36me3

Similar to the findings for H3K36me2, log(RBC folate) was inversely associated with log(H3K36me3) in men (β: -0.19; 95% CI: -0.38, 0.01; $P = 0.06$). Log(plasma folate) was not significantly associated with log(H3K36me3) in either men or women. Among women, both

log(cobalamin) (β: 0.11; 95% CI: 0.00, 0.22; *P* = 0.05) and log(choline) (β: 0.35; 95% CI: 0.08, 0.62; *P* = 0.01) were positively associated with log(H3K36me3). There was a similar trend for log(choline) and log(H3K36me3) among men (β: 0.17; 95% CI: -0.09, 0.43; *P* = 0.21). Log(Hcys) was inversely associated with log(H3K36me3) in men (β: -0.15; 95% CI: -0.29, -0.02; *P* = 0.03), but this was not significant among women.

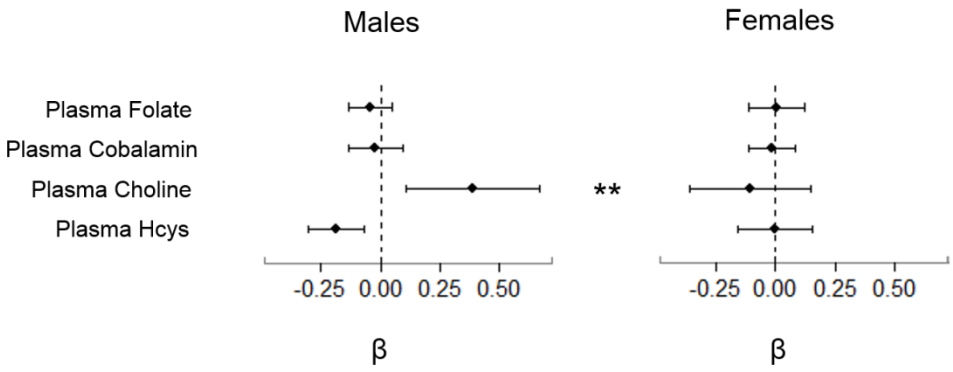
H3K79me2

Although not significant, both log(RBC folate) (β: -0.18; 95% CI: -0.38, 0.02; *P* = 0.08) and log(plasma folate) (β: 0.11; 95% CI: -0.28, 0.05; *P* = 0.18) were inversely related to log(H3K79me2) among women. There was a similar trend for log(RBC folate) among men (β: -0.17; 95% CI: -0.43, 0.08; *P* = 0.18). Additionally, both log(choline) (β: 0.28; 95% CI: -0.06, 0.62; *P* = 0.10) and log(cobalamin) (β: 0.23; 95% CI: 0.09, 0.37; *P* < 0.01) were positively associated with log(H3K79me2) in women, but not men, and the associations between log(cobalamin) and log(H3K79me2) differed by sex (*P* < 0.05). Log(Hcys) concentrations were not significantly associated with log(H3K79me2) in either men or women.

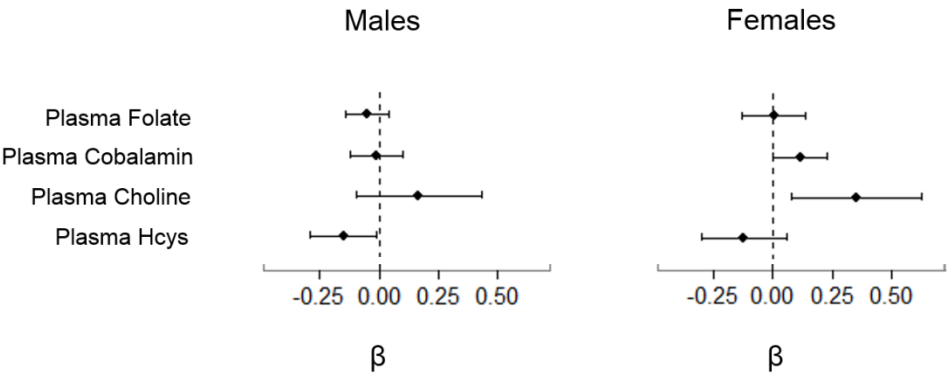
Sensitivity Analyses

Associations between OCM indices and PTHMs were very similar after additionally adjusting for BMI (**Supplemental Material, Table S4**) and measures of arsenic exposure (data not shown). Associations were also similar when OCM indices were examined individually, rather than in the same model, although some of the effects were slightly attenuated (**Supplemental Material, Table S5**).

A **H3K36me2**



B **H3K36me3**



C **H3K79me2**

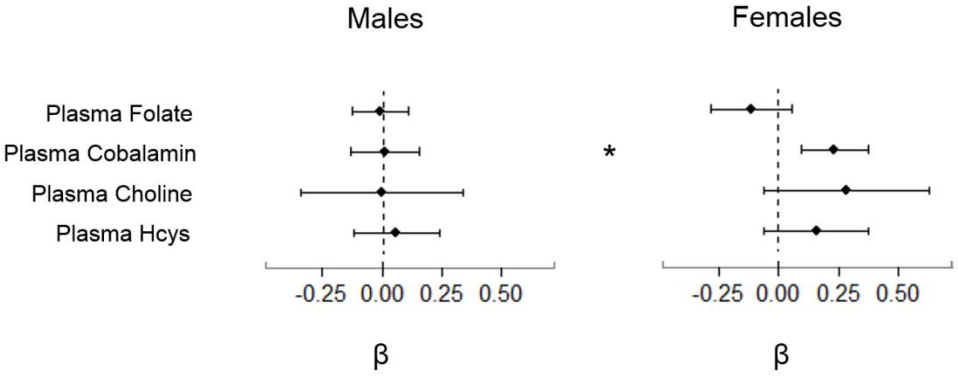


Figure 1. Sex-specific associations between OCM indices and PTHMs in FACT participants Estimated regression coefficients and 95% confidence intervals for associations between each OCM index and (**A**) H3K36me2, (**B**) H3K36me3, and (**C**) H3K79me2 are shown separately by sex. The dashed line represents the null ($\beta = 0$). Associations with confidence intervals that do not cross the null are statistically significant ($P < 0.05$). Asterisks (*$P < 0.05$, **$P < 0.01$) indicate sex differences with *P*-values calculated from the Wald test. OCM indices were natural log-transformed and were included simultaneously in models. Models were adjusted for age, education, TV ownership, and log(bSe). Analyses for H3K36me3 and H3K79me2 in men were additionally adjusted for cigarette smoking status. H3K36me2 was inverse-transformed and was modeled using a generalized linear model with an inverse-link function. H3K36me3 and H3K79me2 were natural log transformed and were modeled using linear models. Sample sizes for the main analyses were as follows H3K36me2: $n = 159$ for men, $n = 159$ for women; H3K36me3: $n = 154$ for men, $n = 152$ for women; H3K79me2: $n = 162$ for men, $n = 159$ for women. Abbreviations used: bSe, blood selenium; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; Hcys, homocysteine; OCM, one-carbon metabolism; PTHM, posttranslational histone modification

Effect of FA Supplementation on PTHMs

Baseline characteristics of participants with PTHM measures were generally comparable between the 400 µg FA and placebo treatment arms, except for uCr, which was significantly higher in the 400 µg FA group (**Supplemental Material, Table S6**). However, uCr was not associated with any of the PTHMs at baseline, nor was it associated with the intra-person change in any of the PTHMs.

Compared with placebo, FA supplementation (400 µg/day for 12 weeks) did not alter any of the PTHMs (**Table 1**). In sex-stratified analyses, there was also no effect of FA compared with placebo on any of the PTHMs (Table 1).

Table 1. Within-person change[a] in PTHM from baseline to week 12 in FACT participants by treatment arm

PTHM	400 µg FA	Placebo	Test for group difference
	Median (IQR)	Median (IQR)	*P*[b]
All Participants			
H3K36me2[c]	-0.05 (-0.39, 0.11)	-0.15 (-0.43, 0.11)	0.39
H3K36me3[d]	0.02 (-0.28, 0.27)	0.02 (-0.23, 0.30)	0.77
H3K79me2[e]	-0.06 (-0.28, 0.14)	-0.05 (-0.24, 0.04)	0.80
Males			
H3K36me2[f]	-0.06 (-0.31, 0.11)	-0.07 (-0.44, 0.16)	0.75
H3K36me3[g]	0.00 (-0.28, 0.23)	0.05 (-0.23, 0.47)	0.44
H3K79me2[h]	-0.03 (-0.19, 0.21)	-0.03 (-0.28, 0.09)	0.70
Females			
H3K36me2[i]	-0.05 (-0.44, 0.10)	-0.17 (-0.37, 0.04)	0.35
H3K36me3[j]	0.04 (-0.31, 0.28)	0.02 (-0.22, 0.12)	0.62
H3K79me2[k]	-0.13 (-0.47, 0.08)	-0.05 (-0.18, 0.04)	0.46

Abbreviations used: FA, folic acid; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; IQR, inter-quartile range; PTHM, posttranslational histone modification

[a]Median (IQR)
[b]*P* from Wilcoxon rank-sum test for treatment group difference
[c]400 µg FA *n* = 103, Placebo *n* = 56
[d]400 µg FA *n* = 98, Placebo *n* = 55
[e]400 µg FA *n* = 97, Placebo *n* = 56
[f]400 µg FA *n* = 52, Placebo *n* = 27
[g]400 µg FA *n* = 50, Placebo *n* = 28
[h]400 µg FA *n* = 50, Placebo *n* = 29
[i]400 µg FA *n* = 51, Placebo *n* = 29
[j]400 µg FA *n* = 48, Placebo *n* = 27
[k]400 µg FA *n* = 47, Placebo *n* = 27

DISCUSSION:

Since lysine histone methyltransferases are dependent on SAM, many PTHMs are sensitive to nutritional methyl donors and other OCM indices [9, 25, 26, 39-42]. However, few studies have examined the influences of nutritional methyl donors on PTHMs in human populations. This has been particularly understudied in populations with a high prevalence of HHcys or chronic exposure to environmental carcinogens, such as arsenic. In this study of arsenic-exposed Bangladeshi adults, we observed sex-dependent associations between several OCM indices and three PTHMs (H3K36me2, H3K36me3, and H3K79me2), which were selected because they are dysregulated in cancers [19-24] and are altered by nutritional methyl donors and/or arsenic in experimental studies [25-28].

Although the nutritional methyl donors and cofactors examined in this study were generally positively associated with the PTHMs examined, this was not the case for folate. We had hypothesized *a priori* that folate would be positively associated with PTHMs. However, plasma folate was not associated with these PTHMs, and supplementation with 400 μg FA/day for 12 weeks did not alter them. In contrast, among men we observed inverse relationships between RBC folate and PTHMs, similar to a previous *in vitro* study, which found that mild folate deficient conditions induced higher global levels of H3K36me2 in a prostate cancer cell line [25]. There is evidence that tetrahydrofolate may facilitate histone demethylation by accepting one-carbon groups as they are removed from histones [43, 44]. Thus, folate may have dual roles in regulating PTHMs, complicating predictions of its net effects on these marks. Although we observed discrepancies between the findings for RBC and plasma folate, this may not be surprising, as RBC folate is a better indicator of long-term folate status. Since both RBCs

and PBMCs originate from hematopoietic stem cells, RBC folate may also better reflect folate status during the epigenetic programming of PBMC progenitor cells.

There are several possible explanations for the sex differences observed in our study. First, consistent with other reports [4, 5], plasma choline, betaine, and Hcys concentrations were higher among men, and men were more likely to be folate deficient. Given that long range allosteric interactions normally regulate SAM concentrations, it is possible that PTHMs are only perturbed under conditions of nutritional deficiencies or excess. There are also underlying sex differences in the OCM pathway. For example, phosphatidylethanolamine *N*-methyltransferase, which catalyzes phosphatidylcholine synthesis, is up-regulated by estrogen [45]. Furthermore, there are sex differences in epigenetic regulation. For example, many histone methyltransferases and histone demethylases bind to androgen receptor [46], and the presence or absence of androgen has been shown to influence the particular PTHMs targeted by these enzymes [47]. Additionally, some histone demethylase genes reside exclusively on the Y chromosome [48].

Both epidemiological and animal studies have shown that susceptibility to arsenic toxicity differs by sex, with some outcomes preferentially affecting males and others females. For example, men are more susceptible to developing cancers of the skin, liver, and bladder after chronic exposure to arsenic [49-51]. It is possible that differential effects of arsenic and OCM indices on epigenetic marks, such as PTHMs, contribute to these differences. We have previously observed that arsenic exposure is associated with DNA methylation and PTHMs in a sex-dependent manner [15-17, 52]. The findings from this study suggest that some of the OCM indices analyzed, e.g. choline and cobalamin, also influence certain PTHMs differentially by sex.

Our findings may also have important implications for targeted clinical interventions. While the majority of epigenetic therapies are still being evaluated *in vitro* and/or in preclinical

studies, others are in early clinical trials or have already been approved by the Food and Drug Administration [53]. Many of these epigenetic therapies target the OCM pathway. For example, EPZ-5676, which is currently in a Phase I trial for the treatment of MLL-rearranged leukemia, is a SAM-competitive inhibitor of DOT1L, a histone methyltransferase which targets H3K79 [54]. Thus, it is possible that nutritional factors and/or the use of supplements that influence SAM concentrations could counteract the effects of EPZ-5676 and other epigenetic drugs. By analogy, there is evidence that antifolates, such as methotrexate, are more effective in patients with low baseline folate concentrations and less effective in individuals taking FA supplements (reviewed in [55]). Thus, a better understanding of how methyl donors influence the epigenetic machinery may ultimately inform therapeutic approaches that target specific epigenetic marks.

The data reported herein suggest that OCM indices influence PTHMs in a sex-dependent manner and further demonstrate that FA supplementation, at least at a dose of 400 μg/day for 12 weeks, does not influence PTHMs in PBMCs. Nevertheless, we cannot rule out the possibility that PTHMs in other target tissues, or other PTHMs, may have been influenced by FA supplementation. Thus, understanding the effects of FA and other nutritional donors on PTHMs in human populations merits additional study.

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Authors' contributions to manuscript: M.V.G designed the study. C.G.H. wrote the manuscript with feedback from M.V.G, M.N.H., X.L., and J.H.G. Statistical analyses were conducted by C.G.H. with feedback from X.L. PTHMs were measured by C.G.H based on a method developed in the laboratory of M.C. Folate and Hcys were measured by V.I., in the laboratory of M.V.G.

Plasma choline and betaine were measured by O.M. in the laboratory of M.A.C. Arsenic and bSe were measured by A.M.L-L in the laboratory of J.G.H. Field work was completed by F.P., A.B.S., H.S., N.U., and T.I.

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CHAPTER SEVEN SUPPLEMENTAL MATERIAL

Table S1. General baseline characteristics by sex for FACT participants with PTHM measures

	All Participants (*n* = 324)	**Males (*n* = 162)**	**Females (*n* = 162)**	
Characteristic	Median (range)	Median (range)	Median (range)	*P*[a]
Age (y)	39 (24-54)	42 (25-54)	37 (24-54)	<0.01
BMI (kg/m^2)[b]	19.2 (13.9-31.6)	18.7 (15.4-27.9)	20.0 (13.9-31.6)	<0.01
Blood Arsenic (µg/L)	8.7 (1.0-80.2)	9.5 (2.5-52.0)	7.9 (1.0-80.2)	0.04
bSe (µg/L)	134 (74-203)	135 (90-191)	132 (74-203)	0.16
RBC Folate (nmol/L)[c]	451 (148-3800)	434 (155-1150)	461 (148-3800)	0.34
Plasma Folate (nmol/L)	12 (3-120)	12 (3-120)	13 (4-42)	0.10
Plasma Cobalamin (pmol/L)	215 (58-871)	217 (58-610)	213 (58-871)	0.49
Plasma Choline (µmol/L)	11.0 (6.0-20.0)	11.9 (6.0-20.0)	10.7 (6.0-19.5)	<0.01
Plasma Betaine (µmol/L)	42.8 (14.3-98.4)	47.0 (20.9-89.4)	37.3 (14.3-98.4)	<0.01
Plasma Hcys (µmol/L)	11.4 (4-102)	14 (6-102)	9 (4-56)	<0.01
H3K36me2[d] (% of total H3)	1.44 (0.68-6.87)	1.45 (0.68-4.00)	1.43 (1.00-6.87)	0.66
H3K36me3[e] (% of total H3)	1.61 (0.48-6.44)	1.56 (0.48-4.09)	1.63 (0.52-6.44)	0.10
H3K79me2[f] (% of total H3)	1.27 (0.29-9.46)	1.26 (0.29-9.46)	1.29 (0.29-9.41)	0.96
Folate Deficient[g] (%)	23.2	28.4	17.9	0.03
Cobalamin Deficient[h] (%)	24.4	24.1	24.7	0.90
HHcys (%)[i]	40.7	62.4	19.1	<0.01
Ever Smoked Cigarette (%)	28.8	56.2	1.2	<0.01[j]

Ever Used Betel Nut (%)	26.9	29.0	24.8	0.40
Education >5 y (%)	22.5	21.0	24.1	0.51
Own TV (%)	38.3	32.7	43.8	0.04

Abbreviations used: BMI, body mass index; bSe, blood selenium; FACT, Folic Acid and Creatine Trial; H3, histone H3; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; Hcys, homocysteine; HHcys, hyperhomocysteinemia; PTHM, posttranslational histone modification; RBC, red blood cell

[a]*P* was from Wilcoxon rank-sum test and Chi square test for sex difference for continuous and categorical variables, respectively
[b]Whole sample, *n* = 315; Men, *n* = 160; Women, *n* = 155
[c]Whole sample, *n* = 250; Men, *n* = 125 ; Women, *n* = 125
[d]Whole sample, *n* = 318; Men, *n* = 159; Women, *n* = 159
[e]Whole sample, *n* = 306; Men, *n* = 154; Women, *n* = 152
[f]Whole sample, *n* = 321; Men, *n* = 162; Women, *n* = 159
[g]Plasma folate <9 nmol/L
[h]Plasma cobalamin <151 pmol/L
[i]Plasma Hcys >13 μmol/L
[j]*P* was from Fisher's exact test, since there were only two female smokers

Table S2. Baseline characteristics of FACT participants with vs. without PTHM measures

	PTHMs Measured (*n* = 324)	**PTHMs Not Measured (*n* = 285)**	
Characteristic	Median (range)	Median (range)	*P*[a]
Age (y)	39 (24-54)	37 (24-55)	0.05
BMI (kg/m^2)[b]	19.2 (13.9-31.6)	19.5 (14.3-27.6)	0.44
Blood Arsenic (µg/L)	8.7 (1.0-80.2)	8.8 (1.8-35.0)	0.38
bSe (µg/L)	134 (74-203)	137 (89-226)	0.10
RBC Folate (nmol/L)[c]	451 (148-3800)	435 (172-1067)	0.55
Plasma Folate (nmol/L)	12 (3-120)	15 (4-168)	<0.01
Plasma Cobalamin (pmol/L)	215 (58-871)	216 (51-864)	0.58
Plasma Choline (µmol/L)	11.0 (6.0)	11.9 (6.4-20.1)	0.01
Plasma Betaine (µmol/L)	43 (14-98)	45 (12-116)	0.05
Plasma Hcys (µmol/L)	11 (4-102)	11 (5-107)	0.42
Folate Deficient[d] (%)	23.1	15.8	0.03
Cobalamin Deficient[e] (%)	24.4	23.5	0.88
HHcys[f] (%)	40.7	36.1	0.28
Ever Smoker (%)	28.8	25.3	0.38
Education >5 y (%)	22.5	21.4	0.12
Own TV (%)	38.3	50.9	<0.01

Abbreviations used: BMI, body mass index; bSe, blood selenium; FACT, Folic Acid and Creatine Trial; Hcys, homocysteine; HHcys, hyperhomocysteinemia; PTHM, posttranslational histone modification; RBC, red blood cell;

[a]*P* was from by Wilcoxon rank-sum test and Chi Square test for difference between those with vs. without PTHM measures for continuous and categorical variables, respectively
[b]*n* = 315 in those with PTHMs measured, *n* = 284 in those without PTHMs measured
[c]*n* = 250 in those with PTHMs measured, *n* = 146 in those without PTHMs measured
[d]Plasma folate <9 nmol/L
[e]Plasma cobalamin <151 pmol/L
[f]Plasma Hcys >13 µmol/L

Table S3. Baseline characteristics of FACT participants with vs. without RBC folate measures

	RBC Folate Measured (*n* = 250)	**RBC Folate Not Measured (*n* = 74)**	
Characteristic	Median (Range)	Median (Range)	*P*[a]
Age (y)	39 (24-54)	38 (26-54)	0.52
BMI (kg/m^2)[b]	19.1 (13.9-31.6)	19.7 (14.8-26.9)	0.73
Plasma Folate (nmol/L)	13 (3-120)	12 (3-50)	0.81
Plasma Cobalamin (pmol/L)	209 (58-634)	247 (69-871)	0.04
Plasma Choline (µmol/L)	11.0 (6.0-19.5)	11.3 (6.0-20.0)	0.64
Plasma Betaine (µmol/L)	43.2 (14.3-89.4)	40.5 (19.1-98.4)	0.49
Plasma Hcys (µmol/L)	12 (5-102)	11 (4-31)	0.21
Blood Arsenic (µg/L)	8.8 (1.0-80.2)	8.2 (2.2-53.9)	0.53
bSe (µg/L)	134 (74-203)	134 (92-186)	0.39
uCr (mg/dL)	46 (6-233)	59 (7-252)	0.04
H3K36me2[c] (% of total H3)	1.46 (0.68-6.87)	1.39 (1.01-5.98)	0.41
H3K36me3[d] (% of total H3)	1.61 (0.48-6.00)	1.61 (0.54-6.44)	0.45
H3K79me2[e] (% of total H3)	1.25 (0.29-9.46)	1.32 (0.70-5.87)	0.52
Folate Deficient (%)[f]	23.6	21.6	0.72
Cobalamin Deficient (%)[g]	26.0	18.9	0.21
HHcys (%)[h]	41.6	37.8	0.56
Male (%)	50.0	50.0	0.99
Ever Smoker (%)	26.9	35.1	0.17

Education >5 y (%)	22.8	21.6	0.83
Own TV (%)	41.6	27.0	0.02

Abbreviations used: BMI, body mass index; bSe, blood selenium; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; Hcys, homocysteine; HHcys, hyperhomocysteinemia; uCr, urinary creatinine

[a]*P* was from Wilcoxon rank-sum test and Chi Square test for difference between those with vs. without RBC folate measures for continuous and categorical variables, respectively
[b]*n* = 245 for those with RBC folate measures, *n* = 70 for those without RBC folate measures
[c]*n* = 245 for those with RBC folate measures, *n* = 73 for those without RBC folate measures
[d]*n* = 239 for those with RBC folate measures, *n* = 67 for those without RBC folate measures
[e]*n* = 249 for those with RBC folate measures, *n* = 72 for those without RBC folate measures
[f]Plasma folate <9 nmol/L
[g]Plasma cobalamin <151 pmol/L
[h]Plasma Hcys >13 µmol/L

Table S4. Associations[a] (β (95% CI)) between OCM indices and PTHMs by sex in FACT participants, comparing models additionally adjusting for BMI

	H3K36me2		H3K36me3		H3K79me2	
	Males[b]	Females[c]	Males[d]	Females[e]	Males[f]	Females[g]
Plasma Folate[h]	-0.02 (-0.12, 0.07)	-0.01 (-0.13, 0.11)	-0.04 (-0.13, 0.05)	-0.02 (-0.15, 0.11)	-0.02 (-0.13, 0.10)	-0.10 (-0.27, 0.06)
Plasma Folate[i]	-0.04 (-0.14, 0.05)	0.00 (-0.11, 0.12)	-0.05 (-0.14, 0.04)	0.00 (0.13, 0.14)	-0.01 (-0.12, 0.11)	-0.11 (-0.28, 0.05)
Plasma Folate[j]	-0.05 (-0.14, 0.05)	0.00 (-0.12, 0.12)	-0.05 (-0.14, 0.04)	-0.01 (-0.14, 0.13)	-0.01 (-0.13, 0.11)	-0.12 (-0.29, 0.04)
Plasma Folate[k]	-0.04 (-0.14, 0.05)	0.00 (-0.12, 0.12)	-0.05 (-0.13, 0.04)	-0.01 (-0.14, 0.13)	-0.02 (-0.13, 0.10)	-0.12 (-0.29, 0.04)
RBC Folate[h]	-0.30 (-0.49, -0.10)**	-0.08 (-0.21, 0.05)	-0.13 (-0.31, 0.05)	0.03 (-0.14, 0.19)	-0.08 (-0.34, 0.17)	-0.18 (-0.38, 0.02)[#]
RBC Folate[i]	-0.23 (-0.41, -0.05)*	-0.08 (-0.21, 0.04)	-0.19 (-0.38, 0.01)[#]	0.03 (-0.13, 0.20)	-0.17 (-0.43, 0.08)	-0.18 (-0.38, 0.02)[#]
RBC Folate[j]	-0.23 (-0.41, -0.05)*	-0.07 (-0.20, 0.06)	-0.19 (-0.38, 0.01)[#]	0.04 (-0.13 0.20)	-0.17 (-0.43, 0.08)	-0.16 (-0.35, 0.04)
RBC Folate[k]	-0.24 (-0.42, -0.05)*	-0.07 (-0.20, 0.06)	-0.22 (-0.41, -0.02)*	0.04 (-0.13, 0.20)	-0.15 (-0.41, 0.11)	-0.15 (-0.35, 0.04)
Cobalamin[h]	0.00 (-0.11, 0.11)	-0.01 (-0.13, 0.11)	-0.01 (-0.12, 0.10)	0.12 (0.01, 0.23)*	-0.03 (-0.17, 0.11)	0.25 (0.11, 0.39)**
Cobalamin[i]	-0.02 (-0.13, 0.09)	-0.01 (-0.11, 0.12)	-0.01 (-0.12, 0.10)	0.11 (0.00, 0.22)*	0.01 (-0.14, 0.15)	0.23 (0.09, 0.37)**
Cobalamin[j]	-0.01 (-0.12, 0.10)	-0.02 (-0.12, 0.08)	0.00 (-0.11, 0.11)	0.12 (0.01, 0.24)*	0.00 (-0.14, 0.15)	0.23 (0.09, 0.37)**
Cobalamin[k]	-0.01 (-0.13, 0.10)	-0.02 (-0.12, 0.08)	-0.01 (-0.12, 0.10)	0.13 (0.01, 0.24)*	0.02 (-0.13, 0.16)	0.23 (0.09, 0.37)**
Choline[h]	0.30 (0.01, 0.60)*	-0.08 (-0.33, 0.18)	0.18 (-0.08, 0.43)	0.37 (0.10, 0.64)**	0.06 (-0.27, 0.39)	0.29 (-0.05, 0.63)
Choline[i]	0.39 (0.11, 0.66)**	-0.11 (-0.36, 0.14)	0.17 (-0.09, 0.43)	0.35 (0.08, 0.62)*	0.00 (-0.34, 0.33)	0.28 (-0.06, 0.62)
Choline[j]	0.42 (0.14, 0.70)**	-0.13 (-0.38, 0.13)	0.21 (-0.04, 0.47)	0.36 (0.09, 0.64)*	0.00 (-0.34, 0.34)	0.20 (-0.14, 0.55)

Choline[k]	0.42 (0.14, 0.70)**	-0.16 (-0.43, 0.10)	0.17 (-0.09, 0.43)	0.33 (0.04, 0.62)*	0.03 (-0.31, 0.37)	0.16 (-0.20, 0.51)
Hcys[h]	-0.18 (-0.29, -0.06)**	-0.02 (-0.17, 0.13)	-0.15 (-0.28, -0.02)*	-0.14 (-0.31, 0.03)	0.08 (-0.10, 0.25)	0.18 (-0.03, 0.39)
Hcys[i]	-0.19 (-0.30, -0.07)**	0.00 (-0.16, 0.15)	-0.15 (-0.29, -0.02)*	-0.12 (-0.30, 0.05)	0.06 (-0.12, 0.23)	0.16 (-0.06, 0.38)
Hcys[j]	-0.19 (-0.31, -0.07)**	0.00 (-0.16, 0.16)	-0.15 (-0.28, -0.02)*	-0.15 (-0.32, 0.03)	0.06 (-0.12, 0.24)	0.17 (-0.05, 0.39)
Hcys[k]	-0.19 (-0.31, -0.07)**	0.00 (-0.15, 0.16)	-0.15 (-0.28, -0.02)*	-0.14 (-0.32, 0.04)	0.06 (-0.12, 0.24)	0.18 (-0.04, 0.40)

Abbreviations used: BMI, body mass index; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; OCM, one-carbon metabolism; PTHM, posttranslational histone modification; RBC, red blood cell

[a]Models include all nutritional indices (except for RBC folate) simultaneously; RBC folate models were run separately but included plasma cobalamin, choline, and Hcys in models. All nutritional indices are natural log-transformed.
[b]n = 159 for models [a] and [b], n = 157 for models [c] and [d]; n = 122 for all RBC folate models
[c]n = 159 for models [a] and [b], n = 152 for models [c] and [d]; n = 123 for all RBC folate models
[d]n = 154 for models [a] and [b], n = 152 for models [c] and [d]; n = 121 for all RBC folate models
[e]n = 152 for models [a] and [b], n = 146 for models [c] and [d]; n = 118 for RBC folate models [a] and [b], n = 114 for RBC folate models [c] and [d]
[f]n = 162 for models [a] and [b], n = 160 for models [c] and [d]; n = 125 for all RBC folate models
[g]n = 159 for models [a] and [b], n = 153 for models [c] and [d]; n = 124 for RBC folate models [a] and [b], n = 120 for RBC folate models [c] and [d]
[h]Unadjusted Model
[i]Main Model (adjusted for age, education, TV ownership, and log(bSe); for H3K36me3 and H3K79me2 in men, these models were additionally adjusted for cigarette smoking status)
[j]Main Model (excluding those without BMI measures)
[k]Main Model (additionally adjusted for log(BMI))
[#]$P < 0.10$, *$P < 0.05$, **$P < 0.01$

Table S5. Associations[a] (β (95% CI)) between OCM indices and PTHMs by sex in FACT participants, comparing nutrients in model alone vs. included simultaneously

	H3K36me2		H3K36me3		H3K79me2	
	Males[b]	Females[c]	Males[d]	Females[e]	Males[f]	Females[g]
Plasma Folate[h]	-0.04 (-0.14, 0.05)	0.00 (-0.11, 0.12)	-0.05 (-0.14, 0.04)	0.00 (0.13, 0.14)	-0.01 (-0.13, 0.11)	-0.11 (-0.28, 0.05)
Plasma Folate[i]	-0.01 (-0.11, 0.09)	0.00 (-0.11, 0.11)	-0.02 (-0.11, 0.07)	0.05 (-0.07, 0.18)	-0.01 (-0.12, 0.10)	-0.13 (-0.29, 0.04)
RBC Folate[h]	-0.23 (-0.41, -0.05)*	-0.08 (-0.21, 0.04)	-0.19 (-0.38, 0.01)[#]	0.03 (-0.13, 0.20)	-0.17 (-0.43, 0.08)	-0.18 (-0.38, 0.02)[#]
RBC Folate[i]	-0.11 (-0.30, 0.08)	-0.08 (-0.21, 0.03)	-0.08 (-0.26, 0.10)	0.05 (-0.11, 0.21)	-0.15 (-0.38, 0.08)	-0.16 (-0.36, 0.04)
Cobalamin[h]	-0.02 (-0.13, 0.09)	-0.01 (-0.11, 0.12)	-0.01 (-0.12, 0.10)	0.11 (0.00, 0.22)*	0.01 (-0.14, 0.15)	0.23 (0.09, 0.37)**
Cobalamin[i]	0.01 (-0.11, 0.13)	0.00 (-0.10, 0.09)	0.00 (-0.11, 0.11)	0.10 (-0.01, 0.21)[#]	0.00 (-0.14, 0.14)	0.19 (0.05, 0.33)**
Choline[h]	0.39 (0.11, 0.66)**	-0.11 (-0.36, 0.14)	0.17 (-0.09, 0.43)	0.35 (0.08, 0.62)*	0.01 (-0.34, 0.33)	0.28 (-0.06, 0.62)
Choline[i]	0.37 (0.08, 0.66)*	-0.10 (-0.34, 0.14)	0.17 (-0.09, 0.43)	0.28 (0.01, 0.54)*	0.00 (-0.33, 0.33)	0.22 (-0.12, 0.56)
Betaine[h]	0.04 (-0.20, 0.27)	0.01 (-0.16, 0.18)	-0.08 (-0.31, 0.15)	0.05 (-0.15, 0.25)	0.12 (-0.17, 0.42)	-0.02 (-0.26, 0.22)
Betaine[i]	0.17 (-0.05, 0.40)	-0.02 (-0.17, 0.14)	0.06 (-0.14, 0.26)	0.17 (-0.01, 0.35)[#]	0.07 (-0.18, 0.32)	0.08 (-0.14, 0.31)
Hcys[h]	-0.19 (-0.30, -0.07)**	0.00 (-0.16, 0.15)	-0.15 (-0.29, -0.02)*	-0.12 (-0.30, 0.05)	0.06 (-0.12, 0.23)	0.16 (-0.06, 0.38)
Hcys[i]	-0.17 (-0.29, -0.05)**	-0.01 (-0.15, 0.13)	-0.14 (-0.27, -0.01)*	-0.12 (-0.28, 0.05)	0.06 (-0.11, 0.23)	0.18 (-0.02, 0.39)

Abbreviations used: FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; Hcys, homocysteine; OCM, one-carbon metabolism; PTHM, posttranslational histone modification; RBC, red blood cell

[a]All models were adjusted for age, education, TV ownership, and log(bSe); for H3K36me3 and H3K79me2 in men, these models were additionally adjusted for cigarette smoking status
[b]n = 159, n = 122 for RBC folate models
[c]n = 159, n = 123 for RBC folate models
[d]n = 154, n = 121 for RBC folate models
[e]n = 152, n = 118 for RBC folate models
[f]n = 162, n = 124 for RBC folate models
[g]n = 159, n = 124 for RBC folate models
[h]All nutrients included simultaneously (RBC folate or plasma folate and cobalamin, choline, and Hcys); models for RBC folate included cobalamin, choline, and Hcys; models for plasma betaine included plasma folate, cobalamin, choline, and Hcys. All nutrients were natural log-transformed.
[i]Nutrient examined individually in models
[#]$P < 0.10$, *$P < 0.05$, **$P < 0.01$

Table S6. Baseline characteristics of FACT participants with PTHM measures at baseline in 400 µg FA and placebo groups

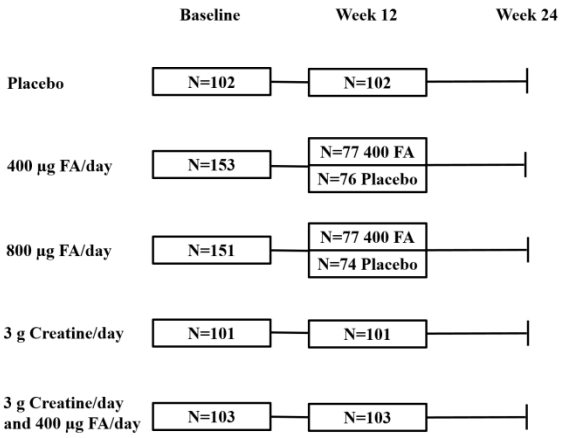
	400 µg FA (*n* = 107)	**Placebo (*n* = 60)**	
Characteristic	Median (Range)	Median (Range)	*P*[a]
Age (y)	38 (25-53)	38 (25-53)	0.29
BMI (kg/m^2)[b]	19.3 (13.9-27.0)	19.5 (15.4-31.6)	0.37
RBC Folate (nmol/L)[c]	424 (148-3800)	472 (186-1159)	0.74
Plasma Folate (nmol/L)	13 (3-120)	13 (3-33)	0.53
Plasma Cobalamin (pmol/L)	213 (58-634)	220 (60-462)	0.76
Plasma Choline (µmol/L)	11.0 (6.0-19.5)	10.9 (6.8-17.3)	0.86
Plasma Betaine (µmol/L)	42.4 (14.3-85.6)	43.4 (14.6-81.2)	0.84
Plasma Hcys (µmol/L)	11.4 (5.2-66.6)	11.7 (5.3-33.1)	0.77
Blood Arsenic (µg/L)	8.4 (2.3-80.2)	8.7 (1.0-34.7)	0.83
bSe (µg/L)	135 (91-195)	136 (90-197)	0.93
uCr (mg/dL)	46 (6-233)	40 (6-121)	0.03
H3K36me2[d] (% of total H3)	1.48 (1.02-6.87)	1.56 (0.68-3.86)	0.61
H3K36me3[e] (% of total H3)	1.62 (0.48-6.00)	1.67 (0.80-4.16)	0.51
H3K79me2[f] (% of total H3)	1.20 (0.59-9.46)	1.16 (0.29-3.46)	0.18
Folate Deficient (%)[g]	26.2	18.3	0.25
Cobalamin Deficient (%)[h]	23.4	26.7	0.63
HHcys (%)[i]	39.3	46.7	0.35

Male (%)	50.5	51.7	0.88
Ever Smoker (%)[j]	28.6	31.7	0.68
Education >5 y (%)	22.4	16.7	0.37
Own TV (%)	42.1	40.0	0.80

Abbreviations used: BMI, body mass index; bSe, blood selenium; FA, folic acid; FACT, Folic Acid and Creatine Trial; Hcys, homocysteine; HHcys, hyperhomocysteinemia; PTHM, posttranslational histone modification; RBC, red blood cell; uCr, urinary creatinine

[a]*P* was from Wilcoxon rank-sum test and Chi-square test for difference between 400 µg FA and placebo groups for continuous and categorical variables, respectively
[b]*n* = 104 for 400 µg FA, *n* = 59 for Placebo group
[c]*n* = 103 for 400 µg FA group
[d]*n* = 105 for 400 µg FA, *n* = 56 for Placebo group
[e]*n* = 102 for 400 µg FA, *n* = 57 for Placebo group
[f]*n* = 106 for 400 µg FA, *n* = 60 for Placebo group
[g]Plasma folate <9 nmol/L
[h]Plasma cobalamin <151 pmol/L
[i]Plasma Hcys >13 µmol/L
[j]*n* = 104 for 400 µg FA

A


Baseline
Week 12
Week 24
Placebo
N=102
N=102
400 µg FA/day
N=153
N=77 400 FA
N=76 Placebo
800 µg FA/day
N=151
N=77 400 FA
N=74 Placebo
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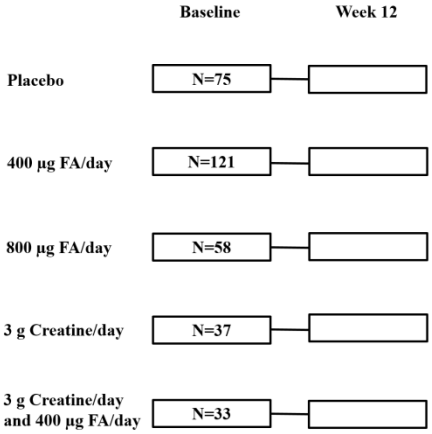
N=101
N=101
3 g Creatine/day
and 400 µg FA/day
N=103
N=103

B


Baseline
Week 12
Placebo
N=75
400 µg FA/day
N=121
800 µg FA/day
N=58
3 g Creatine/day
N=37
3 g Creatine/day
and 400 µg FA/day
N=33

C

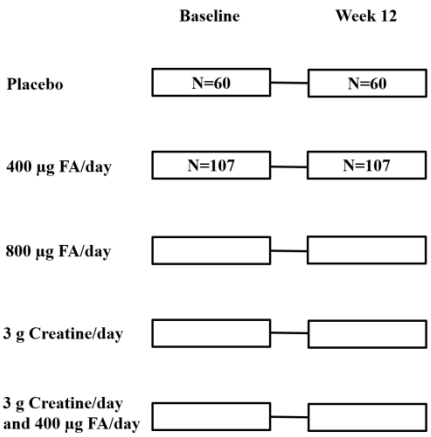
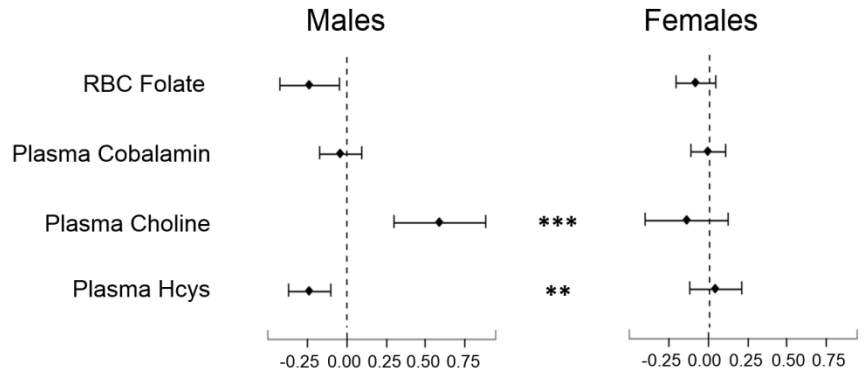
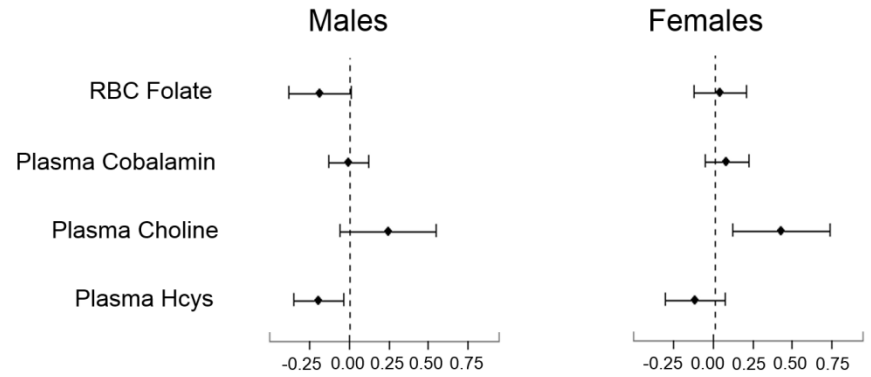

Baseline
Week 12
Placebo
N=60
N=60
400 µg FA/day
N=107
N=107
800 µg FA/day
3 g Creatine/day
3 g Creatine/day
and 400 µg FA/day

Figure S1. FACT design and sampling for current study. **A)** FACT design (primary outcome: blood arsenic). Bangladeshi adults (*n* = 622) who met eligibility criteria for the FACT study were randomized to five treatment arms: placebo, 400 µg folic acid (FA) per day, 800 µg FA per day, 3 g creatine per day, and 3 g creatine + 400 µg FA per day. Participants also received arsenic-removal water filters at baseline to reduce their exposure to arsenic-contaminated drinking water. The study duration was 24 weeks. At week 12, half of the participants in the FA treatment arms were switched to placebo for the duration of the study. The number of participants in each treatment group are shown. 12 participants were dropped from the study for various reasons which have been reported previously [1]. Therefore, a total of 610 participants were included in the primary analyses, which examined the effects of these nutritional interventions on blood arsenic concentrations; the main findings from this trial have been reported [1]. **B)** Participants selected for cross-sectional analyses (secondary outcome: posttranslational histone modifications (PTHMs)). Baseline-collected (i.e., pre-intervention) samples from 324 FACT participants were included in cross-sectional analyses for the current study, which examined associations between one-carbon metabolism (OCM) indices and global levels of PTHMs. All participants from the placebo and 400 µg FA/day treatment arms who had complete information for predictors (OCM indices), PTHMs, and potential confounders were included in these analyses (*n* = 75 for placebo group, *n* = 121 for 400 µg FA/day group); an additional 128 participants with complete information for all relevant variables were randomly selected from the remaining three treatment arms. The distribution of these participants are shown by treatment arm. **C)** Participants selected for examination of FA treatment effects on PTHMs (secondary outcome: PTHMs). Participants with PTHM measures at both time points of interest (baseline and week 12) were included in analyses for the current study to examine the effect of 400 µg FA/day (*n* = 107) vs placebo (*n* = 60) for 12 weeks on PTHMs. Abbreviations used: FA, folic acid; FACT, Folic Acid and Creatine Trial; OCM, one-carbon metabolism; PTHM, posttranslational histone modification

A **H3K36me2**



B **H3K36me3**



C **H3K79me2**

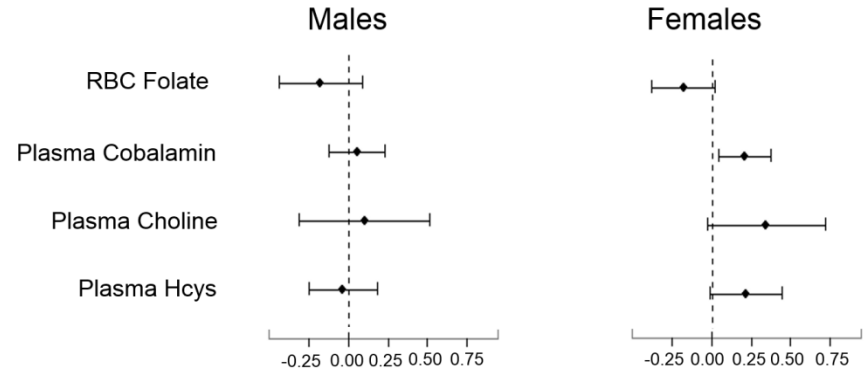


Figure S2. Sex-specific associations between OCM indices and PTHMs in FACT participants with RBC folate measures. Estimated regression coefficients and 95% confidence intervals for associations between each OCM index and (**A**) H3K36me2, (**B**) H3K36me3, and (**C**) H3K79me2 are shown separately by sex. The dashed line represents the null ($\beta = 0$). Associations with confidence intervals that do not cross the null are statistically significant ($P < 0.05$). Asterisks (**$P < 0.01$, ***$P < 0.001$) indicate sex differences with *P*-values calculated from the Wald test. OCM indices were natural log-transformed and were included simultaneously in models. Models were adjusted for age, education, TV ownership, and log(bSe). Analyses for H3K36me3 and H3K79me2 in men were additionally adjusted for cigarette smoking status. H3K36me2 was inverse-transformed and was modeled using a generalized linear model with an inverse-link function. H3K36me3 and H3K79me2 were natural log-transformed and were modeled using linear models. Sample sizes were as follows: H3K36me2: $n = 122$ for men, $n = 123$ for women; H3K36me3: $n = 121$ for men, $n = 118$ for women; H3K79me2: $n = 125$ for men, $n = 124$ for women. Abbreviations used: FACT; Folic Acid and Creatine Trial; H3K36me2, di-methylation at lysine 36 of histone H3; H3K36me3, tri-methylation at lysine 36 of histone H3; H3K79me2, di-methylation at lysine 79 of histone H3; Hcys, homocysteine; OCM, one-carbon metabolism; PTHM, posttranslational histone modification; RBC, red blood cell

CHAPTER SEVEN SUPPLEMENTAL MATERIAL REFERENCES:

1. Peters BA, Hall MN, Liu X, Parvez F, Sanchez TR, van Geen L, et al. Folic Acid and Creatine as Therapeutic Approaches to Lower Blood Arsenic: A Randomized Controlled Trial. Environ Health Perspect. 2015;123(12): 1294-1301.

CHAPTER EIGHT

Conclusions and future directions

A. Summary of main findings

The overarching goal of this dissertation was to gain a better understanding of how nutrients involved in one-carbon metabolism (OCM) influence two mechanisms implicated in arsenic toxicity. The main objectives were 1) to examine the relationships between *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH), and arsenic metabolism, and the potential modifying effects of folate and cobalamin, two nutrients involved in the OCM pathway, and 2) to characterize the effects of arsenic and OCM indices on epigenetic modifications, with a particular focus on posttranslational histone modifications (PTHMs). Since susceptibility to arsenic toxicity differs by sex [1], we further investigated whether the effects of arsenic and OCM indices were sex-dependent. We tested these hypotheses using data from three epidemiological studies in arsenic-exposed Bangladeshi adults: the Folate and Oxidative Stress (FOX) study, the Folic Acid and Creatine Trial (FACT), and the Bangladesh Vitamin E and Selenium Trial (BEST).

Chapter 3 (Specific Aims 1a and 1b).

We hypothesized that 1) SAM would be associated with a ↓ proportion of inorganic arsenical species (%InAs) in blood and urine, 2) the associations between SAM and the proportions of monomethyl (%MMA) and dimethyl (%DMA) arsenical species in blood and urine would be modified by folate and cobalamin nutritional status, and 3) SAH would be associated with an arsenic metabolite profile indicative of reduced methylation capacity (i.e., ↑%InAs, ↑%MMA, ↓%DMA in blood and urine).

Main findings from Chapter 3 (Specific Aims 1a and 1b).

In **Chapter 3**, we observed that among FOX participants, blood SAM was negatively associated with the %InAs in urine, while the associations between SAM and the methylated arsenic metabolites (%MMA and %DMA) differed depending on folate and cobalamin nutritional status. Among individuals who were deficient for both folate and cobalamin, SAM was associated with an arsenic metabolite profile indicative of reduced methylation capacity (i.e., ↑%MMA, ↓%DMA), which has been associated with cancers, cardiovascular disease, and other adverse health outcomes ([2-7] and reviewed in [8]). Since folate and cobalamin play important roles in the regeneration of SAM, these findings suggest that when folate and cobalamin concentrations are low, available SAM is used primarily for the synthesis of MMA, a toxic intermediate of arsenic metabolism, at the expense of DMA, a less toxic metabolite that is rapidly eliminated in urine. Although blood SAH was not associated with any of the arsenic metabolites, plasma homocysteine (Hcys) was associated with an arsenic metabolite profile indicative of reduced methylation capacity (↑%MMA, ↓%DMA), suggesting that plasma Hcys may be a better biomarker of hepatic SAH concentrations than blood SAH itself. This is consistent with a hypothesis by James et al. that Hcys is an "exportable form of SAH" [9]. To our knowledge, this is the first human study to examine the relationships between SAM, SAH, and arsenic metabolites.

In Chapter 3, we also observed decreasing ratios of DMA to MMA concentrations in blood (µg/L) among individuals with increasing concentrations of blood InAs or MMA, suggesting that both InAs and MMA may inhibit the second arsenic methylation step (i.e., the methylation of MMA to DMA). While these findings are associative and do not establish

causality, they support previous experimental studies conducted in primary rat and human hepatocytes, which demonstrated that the production of methylated metabolites, particularly DMA, decrease with exposure to increasing concentrations of InAs [10], and *in silico* models, which predict that MMA inhibits its own methylation, likely via substrate inhibition (Michael Reed and Fred Nijhout, Duke University, personal communication).

Collectively, these findings suggest that individuals deficient for both folate and cobalamin, and individuals exposed to higher concentrations of arsenic, have a reduced capacity to completely methylate InAs to DMA. Consequently, these individuals may be more susceptible to arsenic toxicity.

Chapter 4.

In our early analyses of PTHMs, we identified a specific cleavage product of histone H3 in human peripheral blood mononuclear cells (PBMCs). The objectives of this chapter were to 1) briefly describe and characterize this cleavage product, 2) determine the prevalence of H3 cleavage among PBMC samples, and 3) evaluate the impact of H3 cleavage on the measurement of PTHMs located upstream vs. downstream of H3 cleavage sites.

Main findings from Chapter 4.

In **Chapter 4**, we presented Western blots showing differential cleavage of histone H3 in a subset of human PBMC samples collected from FACT participants. Although H3 cleavage or "clipping" has been observed in many other species and in other human cell types (reviewed in [11-13]), this is the first study to describe H3 cleavage in human PBMCs. We observed extensive H3 cleavage in approximately one third of the PBMC samples examined. Furthermore, we demonstrated that PTHMs located downstream of published H3 cleavage sites [14], such as di-

methylation at lysine 9 of histone H3, are reduced or non-detectable in samples with extensive cleavage. In contrast, PTHMs located upstream of cleavage sites, such as di-methylation at lysine 36 of histone H3 (H3K36me2) and di-methylation at lysine 79 of histone H3 (H3K79me2), were not affected by H3 cleavage.

Currently, it is unclear if H3 cleavage is a biological or methodological phenomenon. A recent publication provides an in-depth description of the difficulties in distinguishing between histone degradation and biological histone "clipping" [11]. We did not observe that age, sex, nutritional status, cigarette smoking status, or most other general characteristics among FACT participants, were associated with the presence or absence of H3 cleavage in PBMCs. However, we did observe that H3 cleavage was significantly more prevalent among those who had ever chewed betel nut, a stimulant and established human carcinogen that is commonly used in parts of Asia, including Bangladesh [15]. Whether or not this relationship is causal merits additional investigation.

Regardless of the underlying cause of H3 cleavage, our findings have important implications for molecular epidemiology studies. Moving forward, Western blot analysis can be used to screen banked or recently collected samples for histone cleavage to determine if it is appropriate to measure certain PTHMs. However, previous studies which did not screen for histone cleavage, and which measured potentially affected PTHMs, should be interpreted with caution.

Chapter 5 (Specific Aims 2a and b).

We hypothesized that arsenic exposure would be associated with global levels of three PTHMs located on histone H3: H3K36me2, H3K79me2, and tri-methylation at lysine 36 of

histone H3 (H3K36me3), measured in PBMCs collected from FACT participants. These PTHMs were selected because they are dysregulated in cancers [16-21] and influenced by arsenic and/or nutritional methyl donors in experimental models [22-25]. We also selected H3K36me2 and H3K79me2 based on our preliminary data on a) lysine demethylase 2B, a histone demethylase which targets H3K36me2, and on b) 5-hydroxymethylcytosine (5-hmC) [26], since H3K79me2 has been shown to regulate the expression of *TET1*, one of the enzymes which converts 5-methylcytosine (5-mC) to 5-hmC [27, 28]. Our candidate PTHMs are not affected by H3 cleavage. Given that previous studies have observed sex-dependent effects of arsenic on DNA methylation [26, 29-31] and other PTHMs [32, 33], we further hypothesized that the associations between arsenic and our candidate PTHMs would differ by sex. We also hypothesized that PTHMs would be stable after the use of arsenic-removal water filters, since epigenetic dysregulation may be one mechanism contributing to the persistent cancer risks observed in populations that were previously exposed to arsenic [34].

Main findings of Chapter 5 (Specific Aims 2a and b).

In **Chapter 5**, we observed that the associations between creatinine-adjusted urinary arsenic (uAs_{Cr}) and H3K36me2 differed significantly by sex. Arsenic exposure was positively associated with global levels of H3K36me2 among men, but not women. Our findings support several previous epidemiological and experimental studies, which have also observed sex-dependent effects of arsenic on epigenetic modifications [26, 29-33], including PTHMs [32, 33]. In particular, these findings parallel our previous observation that arsenic exposure is positively associated with global levels of 5-mC among Bangladeshi men, but not women [26]. Since 5-mC and PTHMs are highly interrelated [35, 36], and since PTHMs are thought to be more labile than 5-mC [35], alterations in H3K36me2 may mediate the sex-dependent effects of arsenic on 5-mC.

This has potential public health implications, as DNA hypomethylation has been associated with an increased risk of developing arsenic-induced skin lesions [37]. Therefore, the potential mediating effects of H3K36me2 and other PTHMs should be examined in future studies.

In Chapter 5 we also observed that H3K36me2 declined simultaneously with the use of arsenic-removal water filters. However, in sex-stratified analyses, this was observed among both men and women and only achieved statistical significance among women. Since we did not have a comparison group that did not receive arsenic-removal water filters, we cannot rule out the possibility that the decline in H3K36me2 was caused by extrinsic factors. The potential reversibility of arsenic-induced alterations in PTHMs, and downstream epigenetic marks such as DNA methylation, will need to be evaluated in future studies. This is a largely understudied research area that is important for understanding the contribution of epigenetic dysregulation to arsenic-induced health outcomes.

Importantly, we did not observe associations between arsenic exposure and either H3K36me3 or H3K79me2, nor did we observe alterations in these marks as a consequence of arsenic-removal, suggesting that arsenic exposure does not perturb these PTHMs in PBMCs. However, we cannot rule out the possibility that arsenic exposure may influence these PTHMs in other target tissues.

Chapter 6 (Specific Aim 2c).

We hypothesized that uAs_{Cr} would be associated with gene-specific DNA methylation and mRNA expression, measured in whole blood and PBMCs, respectively, collected from Bangladeshi adults with arsenicosis. Based on our prior research, we were interested in genes involved in pathways implicated in arsenic toxicity, including OCM, arsenic metabolism,

epigenetic regulation, DNA repair, and tumor suppression [38-43]. We selected a total of 47 relevant candidate genes. Since previous studies have observed sex-dependent effects of arsenic on several epigenetic marks [26, 29-33], we further hypothesized that these associations would differ by sex.

Main findings of Chapter 6 (Specific Aim 2c).

In **Chapter 6**, we observed that uAs_{Cr} was associated with the differential methylation and expression of several genes involved in OCM, epigenetic regulation, DNA repair, and tumor suppression/oncogenesis. Although our findings generally supported previous studies which evaluated many of the same genes, we identified several novel associations. In particular, we observed differential methylation or expression of several genes involved in the OCM pathway, including *MTHFR*, *GAMT*, and *GNMT*, which had not previously been examined in relation to arsenic exposure.

To our knowledge this was the first large (n = 1799) human study to examine the relationships between arsenic exposure and gene expression separately in men and women. Similar to a previous small human study (n = 29) [44], we observed many sex differences. These findings support our observations from Chapter 5 and the findings of previous studies [26, 29-33], which demonstrated that arsenic exposure is associated with epigenetic modifications, including both DNA methylation and PTHMs, in a sex-dependent manner. For many outcomes, susceptibility to arsenic toxicity has been shown to differ by sex [1]. Sex-specific alterations in epigenetic modifications, and corresponding changes in gene expression, may be one mechanism contributing to this.

Chapter 7 (Specific Aims 3a and 3b).

We hypothesized that OCM indices, including folate, cobalamin, choline, and betaine would be associated with higher global levels of histone methylation marks, while Hcys would be inversely associated with these marks. We additionally hypothesized that folic acid (FA) supplementation would increase global levels of certain histone methylation marks, since lysine histone methyltransferases are SAM-dependent enzymes, and several previous studies have observed that global levels of histone methylation marks are increased by FA or reduced by folate deficiency [23, 24, 45, 46]. Since arsenic has been shown to alter PTHMs in a sex-dependent manner [32, 33], and circulating concentrations of OCM indices differ by sex [47, 48], we further hypothesized that these relationships would differ for men and women.

Main findings of Chapter 7 (Specific Aims 3a and 3b).

In **Chapter 7**, we observed sex-dependent associations between several OCM indices and PTHMs. In particular, the associations between choline and H3K36me2, and between cobalamin and H3K79me2, differed significantly between men and women. Positive associations were observed between choline and H3K36me2 among men and between cobalamin and H3K79me2 among women. However, 400 μg FA per day for 12 weeks did not significantly influence any of the PTHMs examined; this finding was consistent with cross-sectional analyses, which did not find that plasma folate was associated with PTHMs in either men or women. Although our findings suggest that FA does not alter PTHMs, we cannot rule out the possibility that a higher dose or a longer duration may alter these PTHMs. It is also possible that other PTHMs, or PTHMs in other target tissues, may have been affected by this dose of FA for 12 weeks.

Nutritional methyl donors have been shown to protect against toxicant-induced epigenetic dysregulation. For example, one study in mice demonstrated that methyl donor supplementation prevents DNA hypomethylation caused by *in utero* exposure to bisphenol A [49]. Folate and hyperhomocysteinemia have also been shown to modify associations between arsenic and global levels of DNA methylation in human populations [26, 50]. Nutritional methyl donors may also protect against or modify arsenic-induced alterations in global PTHMs. However, a more in depth understanding of the effects of nutritional methyl donors on individual PTHMs is needed. Our findings suggest that FA supplementation, at a dose based on the recommended dietary allowance for folate, does not alter global levels of H3K36me2, H3K36me3, or H3K79me2 in PBMCs. However, choline and cobalamin may influence these PTHMs, and in a sex-dependent manner. To rule out potential reverse causality, the effects of these nutrients on PTHMs will need to be confirmed in prospective studies.

B. Future directions

Although our findings begin to fill some of the many gaps in the literature, they also highlight areas that require additional research. In particular, there is a need for studies which 1) examine the relationships between blood SAM and SAH concentrations with their respective concentrations in the liver, 2) evaluate the causes and consequences of histone cleavage, 3) investigate the impact of arsenic-induced alterations in epigenetic modifications on health and disease, and 4) examine the potential role of PTHMs in mediating arsenic-induced alterations in 5-mC and 5-hmC. The latter can be more fully examined in the near future, as global 5-mC and 5-hmC levels have been measured in samples collected from the FACT study and are currently

being analyzed in relation to arsenic exposure. Other possible future directions are outlined below.

1. New and complementary approaches

Combining multiplex laboratory techniques with cluster analysis

Until recently, large epidemiological studies were limited to measuring individual PTHMs. However, multiplex assays are now available which allow for the simultaneous measurement of PTHMs, and in a relatively high throughput capacity. Since it is the combination of PTHMs which ultimately influences chromatin structure, these methods may improve the ability to use PTHMs as biomarkers. Future studies can combine multiplex assays with cluster analysis to group individuals according to their overall epigenetic profile. For example, multiple PTHM measures could be combined with 5-mC and 5-hmC measures. This overall epigenetic profile could then be examined in relation to environmental exposures, such as arsenic, and related health outcomes. Similar methods have been used previously to predict cancer prognosis based on PTHM profiles (e.g., see [51]).

Gene-specific approaches

Although our findings contribute to growing evidence that arsenic and OCM indices alter global levels of PTHMs in a sex-dependent manner [32, 33], there is a dearth of information on their impacts at the gene-specific level, particularly in humans. This is largely due to the expense of available laboratory methods. However, as these methods become more affordable, they will be critical for better understanding the functional consequences of alterations in global PTHMs. In particular, ChIP-seq, which combines chromatin immunoprecipitation with next generation sequencing, and ATAC-seq, which maps transposase-accessible chromatin [52], can be used

such that arsenic exposure and OCM indices can be examined in relation to PTHM levels within particular genes and also in relation to the overall chromatin landscape.

Mathematical models

Our group has previously used mathematical models of the OCM pathway to successfully predict the effect of FA supplementation on blood arsenic concentrations [53]. Recently, DNA methyltransferases have been incorporated into these models [54]. As we learn more about the properties and kinetics of enzymes involved in PTHM regulation (e.g., [55-57]), this information can be incorporated into similar mathematical models to make predictions about how perturbations in the OCM pathway might affect certain PTHMs. These models could then be used to inform the selection of nutrients, doses, and candidate PTHMs to examine in future studies.

2. Discerning the role of exposure timing

Our studies provide compelling evidence that arsenic is associated with PTHMs, DNA methylation, and gene expression in a sex-dependent manner in adults. However, early life exposure to arsenic may have more profound effects on epigenetic modifications. The perinatal period is thought to be particularly vulnerable to epigenetic dysregulation [58], and there is evidence that individuals are more susceptible to arsenic toxicity after early life exposure. For example, while most animal models do not develop cancers after exposure to arsenic in adulthood, *in utero* exposure to arsenic induces tumor development in adult mice, and in a sex-dependent manner [59]. There is also evidence from human populations that the risk of developing cancers and other adverse health outcomes is greater after *in utero* or early life exposure to arsenic [60-62]. However, for some outcomes postnatal exposure to arsenic may be

more important than prenatal exposure. For example, one study observed that early childhood, but not prenatal, exposure to arsenic was associated with a lower attained body weight and length among girls [63].

Few studies have evaluated the importance of exposure timing on arsenic-induced epigenetic dysregulation. One possibility would be to take advantage of a unique study, nested within the Health Effects of Arsenic Longitudinal Study (HEALS) cohort, consisting of adolescents (14-17.5 years) with four different arsenic exposure patterns: 1) low lifetime exposure (water arsenic <10 μg/L), 2) moderate lifetime exposure (10 μg/L< water arsenic <50 μg/L), 3) high lifetime exposure (water arsenic ≥50 μg/L), and 4) high perinatal exposure (water arsenic ≥50 μg/L), but moderate exposure thereafter (water arsenic <50 μg/L). PBMCs have been collected from these adolescents. Thus, epigenetic marks and gene expression can be examined in relation to these four distinct lifetime exposure patterns to ascertain the contribution of early life exposure to arsenic. Animal studies, which can easily control the timing of exposure, can also complement epidemiological studies, such that the contributions of pre- vs. postnatal, or early vs. late life, exposure to arsenic can be evaluated in relation to epigenetic outcomes.

3. Mechanistic studies of sex differences

The mechanisms underlying the sex-dependent effects of arsenic on epigenetics remain largely unknown. In epidemiological studies, it is difficult to distinguish between the contributions of biological sex vs. gender. While it is important to understand the gender-related factors which contribute to sex differences (e.g., differences in co-exposures, such as UV radiation and cigarette smoke), it is also essential to understand the contributions of biological sex. Although animal models of arsenic toxicity have important limitations, they offer many

advantages for studying the role of biological sex. One particularly well-suited model is the four core genotype mouse model (**Figure 1**), which distinguishes between gonadal vs. genetic contributions to biological sex differences [64].

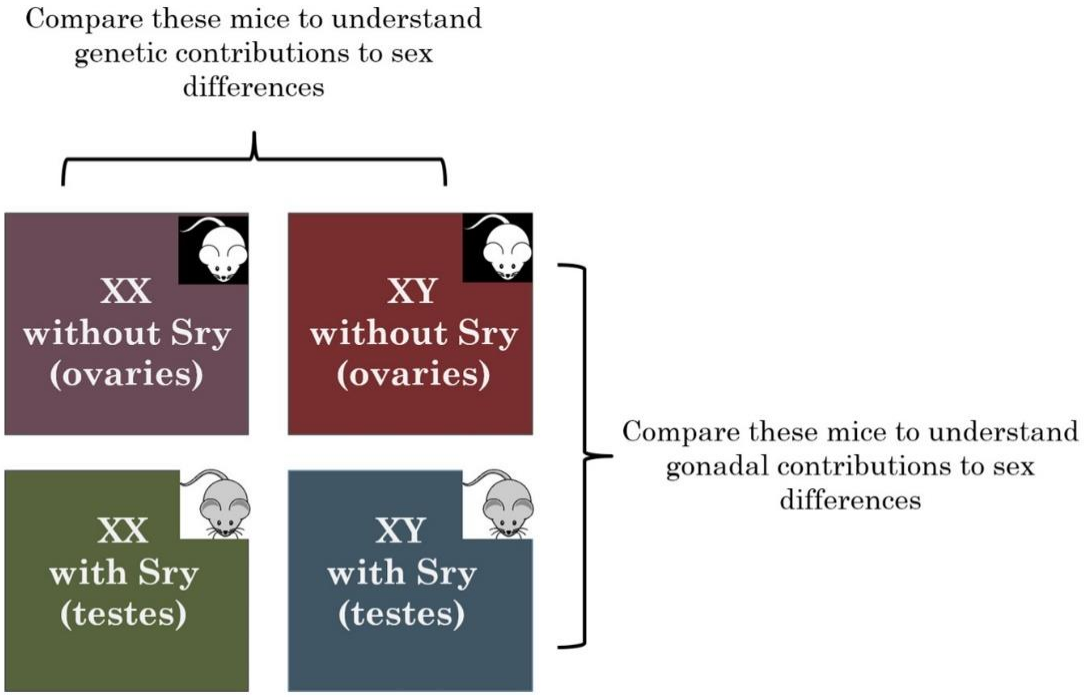


Figure 1. Four core genotype mouse model. This model dissociates genetic sex from gonadal sex. This is accomplished by creating two additional "sex" genotypes by deleting the gene responsible for testis development (*Sry*) from the Y chromosome in male animals (XY) and inserting an *Sry* transgene in an autosome in female animals (XX). The result is four core genotypes (clockwise from top left): 1) genetically female animals (XX) without the *Sry* gene, which develop ovaries (purple), 2) genetically male animals (XY) without the *Sry* gene, which develop ovaries (red), 3) genetically female animals (XX) with the *Sry* transgene, which develop testes (green), and 4) genetically male (XY) animals with the *Sry* gene, which develop testes (blue). Genetic contributions to sex differences can then be determined by comparing animals with the same gonads but with different genotypes (e.g., purple compared with red or green compared with blue). Gonadal contributions to sex differences can be determined by comparing animals with the same genotype but with different gonads (e.g., purple compared with green or red compared with blue). Figure adapted from McCarthy et al. [65].

The effects of arsenic exposure and nutritional interventions on epigenetic modifications, gene expression, and tumor development could be examined in this model to better understand how sex-related genes and/or hormones modify these relationships. Better understanding these mechanisms will be important for developing public health interventions which effectively reduce disease burden among both men and women in arsenic-exposed populations.

C. Conclusions

Collectively, the studies presented in this dissertation provide evidence that 1) deficiencies in folate and cobalamin, and exposure to high arsenic concentrations, reduce arsenic methylation capacity, and 2) arsenic and OCM indices influence PTHMs in a sex-dependent manner. This dissertation also presents the first data demonstrating sex-specific associations between arsenic exposure and gene expression in a large human study and the first evidence from a randomized clinical trial that FA supplementation for 12 weeks, at a dose based on the recommended dietary allowance for folate, does not alter global levels of PTHMs in human PBMCs.

Since arsenic metabolism facilitates urinary arsenic excretion, our data suggest that individuals who are deficient for folate and cobalamin, and individuals exposed to higher concentrations of arsenic, may be particularly susceptible to arsenic toxicity. Our findings also suggest that both arsenic exposure and nutrients involved in the OCM pathway influence PTHMs in a sex-dependent manner, and that arsenic additionally alters gene-specific DNA methylation and mRNA expression differentially by sex. While it is tempting to speculate that these findings may explain some of the sex differences in susceptibility to arsenic toxicity, the clinical implications of these epigenetic alterations will require further investigation. Nevertheless, our

findings contribute to a growing body of evidence that arsenic and OCM indices influence epigenetic modifications, including PTHMs, and that these effects may differ by sex. Our findings are timely, given the recent initiative by the National Institutes of Health to better understand biological sex differences, with the hope that this will reduce inconsistencies between studies and generate findings that will ultimately lead to improvements in health among both men and women [66].

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